[55] G. T. Reul, *Cardiovasc. Dis.*, *4*(1), 61-68 (1977)
[56] H. Kabayashi, M. Tsuzuki, N. Kawano, O. Fakuda, and S. Saito, *Jpn. J. Surg.*, *11*(6), 467–475 (1981).
[57] L. D. Kurtz, South African Patent 7,107,935 (1972); *Chem. Abstr.*, *78*(8), 281 (1973).
[58] L. D. Kurtz, South African Patent 7,204,131 (1973); *Chem. Abstr.* *86*, 250 (1974).
[59] L. D. Kurtz, U.S. Patent 3,862,304 (1975).
[60] M. Stepheneon, U.S. Patent 3,987,797 (1976).
[61] L. V. Lededev, O. F. Mikhailova, L. L. Plotkin, I. S. Kukuruz, Y. K. Krauklis, A. I. Umerenkov, and B. M. Terekhov, *Polim. Med.*, *6*(4), 173–184 (1976).
[62] P. J. Joyce and H. H. Leeven, U.K. Patent Appl. 2,029,224 (1979).
[63] T. J. M. Sinha, Thesis, submitted to I.I.T., Delhi, India, 1983.
[64] J. M. Sinha and P. Vasudevan, Presented at 3rd International Conference on Advances in Stabilization and Controlled Degradation of Polymers, Lucerne, Switzerland, June 1–3, 1981.

JMS—REV. MACROMOL. CHEM. PHYS., C25(3), 325-373 (1985)

Laboratory Synthesis of Polyethylene Glycol Derivatives

J. MILTON HARRIS
Department of Chemistry
University of Alabama in Huntsville
Huntsville, Alabama 35899

 0736-6374/85/2503-0325$3.50/0

I. INTRODUCTION

In recent years, derivatives of polyethylene glycol (PEG) have proven valuable in a variety of diverse chemical and biological endeavors. Such applications include peptide synthesis, phase transfer catalysis, pharmaceutical modification, protein and cell purifications, polymer-bound reagents, and binding assays. Because of the great deal of interest surrounding this subject, this review will describe generally applicable laboratory methods for preparing PEG derivatives from the parent PEG. We have largely restricted discussion to this starting material because most research laboratories interested in applications are not equipped to handle complex ethylene oxide polymerizations used in large-scale industrial preparations and because PEG and some of its ethers and esters are the only commonly available polymeric starting materials. For the purpose of this review, PEG is defined as those polyoxyethylenes having hydroxyl endgroups and a molecular weight of 20,000 daltons or less.

The review is divided into two parts: the synthesis of simple derivatives such as esters, and the synthesis of complex derivatives such as protein conjugates. In general, discussion is limited to polymers having a molecular weight of at least 750 g/mol, although on occasion a reaction of oligomeric ethylene glycols is discussed if the reaction is judged to be relevant and applicable to the larger molecules. An attempt has been made to identify all pertinent papers; the major obstacle to success in this attempt has been difficulty in discovering derivative syntheses in esoteric application papers.

In the interest of brevity, we have adopted the policy when citing a paper of giving only the name of the first author in the text. Full references are given at the end of the text.

As a chemical shorthand, the symbol PEG-X will be used to represent a difunctional PEG derivative $XCH_2CH_2O(CH_2CH_2O)_n CH_2CH_2X$. The symbol M-PEG-X will be used similarly for derivatives of the commonly used monomethyl ether of PEG. More complex derivatives will be explicitly described. Also, note that although reactions of difunctional PEG's frequently give mixtures of mono- and difunctional products and unaltered reagent, we have not indicated these mixtures of products in our equations; the reactions are written as if they go to completion.

General discussion of PEG preparation and properties are available and will not be repeated here [1–5]. A review by Topchieva of biochemical applications of PEG is also available [6].

A. Special Characteristics of PEG-Derivative Synthesis

Most of the synthetic procedures described in this review are classical procedures directly derived from methods well established for preparing organic molecules. The novel aspects of the work generally are encountered in purifications and in the many ingenious applications. Significant difficulty in product purification can arise from the high water-solubility of PEG as this prevents use of the aqueous washes traditional in preparing more typical hydrophobic organic molecules. Also, since there are only one or two reactive sites in the rather large PEG molecule, small masses of low molecular weight impurities can have relatively large molar concentrations that can affect both purifications and reactions.

Water is a particular problem as we have found all commercial PEG's to contain a few tenths of a percent of this reactive impurity (by Karl-Fischer titration). The lower molecular weight polymers have the highest amounts of water (approximately 1.0% for PEG 750). Drying by azeotropic distillation with benzene or toluene reduces water to less than 0.1%. The PEG can be recovered by ether precipitation or used in benzene or toluene solution. Another convenient method for drying PEG is to stir the polymer under vacuum at 110–120°C.

Since the great majority of the syntheses described in this review begin with PEG or M-PEG, it is important to consider the nature of these commercially available materials. PEG's are quite pure materials, having only trace (ppm) amounts of impurities such as dioxane, salts, aldehydes, and free ethylene oxide. Levels of ethylene glycol and diethylene glycol can be as high as 0.2%. PEG's are hygroscopic, and the manufacturing process can introduce some water; consequently, a few tenths of a percent of water are generally present. PEG is nontoxic [7–9] and is cleared by the U.S. Food and Drug Administration for internal use in humans. The molecular weight range is narrow; for example, PEG 8000 will vary by no more than 300 units in either direction. With the exception of some high molecular weights, the polymers are linear. It is difficult to prepare PEG's above 10,000 daltons, and some manufacturers make larger PEG's such as PEG 20,000 by linking two or three PEG 8000 molecules with an aromatic diepoxide. The resulting product is somewhat different from the other PEG's in that it contains a significant amount of branched material and a hydrophobic linking group. The Fluka PEG 20,000 is said to be a linear poly(oxythylene).

The very useful monomethyl ethers of PEG are also available in a wide range of molecular weights. Unfortunately, these materials contain a significant amount (as much as 25%; from size

exclusion chromatography) of PEG without the methoxy end group. This PEG "impurity" results from water present in the polymerization process; under basic conditions hydroxide is produced, which yields PEG upon reaction with ethylene oxide monomer. Also, since the hydroxide-initiated PEG chain can grow at both ends while the methoxide-initiated chain can grow from only one end, the result is a broader molecular weight distribution than that for the PEG's. A laboratory preparation of M-PEG is described in the section on ether synthesis.

1. *Purification*

Significant simplification of product purification can result by taking advantage of PEG's physical properties. In particular, PEG derivatives can be readily precipitated and separated from reagents by adding ethyl ether or hexane, in which PEG is insoluble, to a solution of PEG in an organic solvent such as methylene chloride, benzene, acetone, or acetonitrile. This procedure is especially useful for initial separation of a PEG derivative from a reaction solution. Recrystallizations can be accomplished in ethanol and toluene. Interestingly, the structurally similar poly(oxymethylene) and poly(oxypropylene) have quite different solubilities compared to PEG [4]. For example, PEG is the only one of these polymers to exhibit significant water solubility.

A particularly powerful technique for separating PEG derivatives from low molecular weight reagents and impurities is provided by ultrafiltration or gel chromatography. These techniques are frequently applied when the derivative preparation has been conducted in water where the precipitation technique mentioned above for organic solvents is not applicable. The Pharmacia gel LH-20 is also useful for chromatography in certain organic solvents. Gel filtration and ultrafiltration are also useful for separating unreacted PEG from the product in those cases where the product has a significantly higher molecular weight (e.g., a protein derivative).

Another approach to removing the derivative from an aqueous reaction medium is to extract the derivative into methylene chloride or chloroform. These solvents are unusual in their ability to extract PEG and many of its derivatives from water. For example, in a water—benzene system, PEG favors water by one hundred to one, whereas in a methylene chloride—water system methylene chloride is favored by about seven to one [10]. This partitioning to methylene chloride or chloroform can be reversed to advantage for some derivatives. For example, Ferruti [11], Royer [12], and Fradet [13] have extracted PEG carboxylic acids from water into chloroform at pH below seven, and then extracted the derivative back into water at pH above seven; effective extractive removal of impurities was thus permitted. If the derivative is sufficiently hydrophobic, then, unlike the parent PEG, it may partition in favor of benzene rather than water. For example, we have observed this property with PEG alkyl ethers [14]. Similarly, Sukata has purified PEG-methyl and -ethyl ethers by dissolving them in water at an acidic pH and washing with benzene, then adjusting to a basic pH and extracting the ether from the aqueous medium with benzene [15].

Separations in which the PEG derivative is either much lower or much higher in molecular weight than the impurity can generally be accomplished by the above techniques. In those cases in which there is little molecular weight variation (e.g., in the separation of PEG from its esters or ethers with small acids or alcohols), separation can be much more difficult or impossible; of course, in many instances it is not necessary that PEG be separated from its derivatives. Although this type of separation has not been much explored, it would seem that there are two basic approaches to solution of this problem. The first would be to separate on the basis of charge and the second would be to separate on the basis of specific binding affinity which is not entirely or necessarily charge related. An example of a separation based on charge was given above with the extraction of a PEG carboxylic acid into aqueous base from chloroform [4]. Presumably, such extractions could also be performed with basic PEG's and aqueous acid. Charged PEG's can also be isolated by ion-exchange chromatography. For example, Johansson has isolated anionic PEG dyes by chromatography on DEAE-cellulose [16, 17].

Noncharged PEG alkyl ethers and fatty-acid esters have been separated from PEG by affinity chromatography on octyl-Sepharose [Pharmacia; 10]. This same support should be of use for other derivatives having lipophilic groups although such separations are unknown at present.

Silica gel is potentially useful as an inexpensive and easily used alternative to gel chromatography. For example, we have found that silica gel chromatography with methylene chloride as solvent gives significant fractionation of mono- and di-alkyl PEG ethers and PEG [10, 14]; final purification was provided by gel chromatography.

The well-known ability of PEG to bind metal cations can lead to significant purification problems when certain metallic reagents are used. For example, we attempted the controlled oxidation of PEG to the aldehyde with the highly selective reagent pyridinium chlorochromate, but were not able to remove the product aldehyde from chromium ions by precipitation or chromatography [10]. Alternative synthetic methods were developed before this separation problem could be extensively explored so suitable

purification methods may exist, but this difficulty does point to a potential problem to be aware of when designing synthetic routes.

2. *Derivative Characterization*

Most of the techniques for characterizing molecules are applicable to PEG derivatives, including simple techniques such as melting point and complex ones such as NMR spectroscopy. We will begin with a discussion of chromatography. As mentioned above, gel chromatography provides a powerful means of product purification. This same technique can be used analytically, especially now that the major chromatography firms provide HPLC columns which sort water-soluble polymers on the basis of size [10, 18]. This approach is especially powerful since it provides molecular-weight information in addition to aiding in compound identification. The more common silica and reversed-phase HPLC columns are also very useful, both in detecting low molecular weight impurities [19] and in determining the amount of unreacted PEG in a derivative [14, 20–25]. Thin-layer chromatography is also useful [10, 26–28], especially in detecting low molecular weight impurities, as most eluting solvents give only slight movement of PEG. Gas chromatography can be used similarly for detecting low molecular weight impurities in nonvolatile PEG derivatives [14, 29].

Nuclear magnetic resonance spectroscopy (NMR) is a powerful technique both for determining PEG functional group identity and for determining the extent of functional group conversion from reaction. ^{13}C-NMR is especially powerful as there are large differences in chemical shifts for different carbons. Proton NMR is faster and can be used in certain cases. For example, we have used proton NMR to determine the number of alkane groups in PEG-alkyl ethers [14, 29]. Proton NMR can also be used for qualitative identification of functional groups [30]; thus Mutter [31] monitored the conversion of ester to alcohol by following the disappearance of an ethyl triplet and the appearance of a methyl singlet. Similarly Fradet used proton NMR to identify the methylene adjacent to the carboxyl group in PEG carboxylate [13].

The power of ^{13}C-NMR for PEG analysis has been demonstrated in several recent publications. In a broad-ranging study Bayer has obtained the ^{13}C-spectra of 12 PEG derivatives and has noted the significant and characteristic features of these spectra [32]. The sensitivity of the method was shown by the detection of as little as 2% unsubstituted PEG in the presence of other derivatives. Similarly, Ziegast has shown that the degree of conversion of PEG to PEG amine can be followed readily by ^{13}C-NMR [33]. Barelle has obtained ^{13}C-spectra for a series of oligo(oxyethylene) glycols and their oxidation products [34]. Finally, ^{13}C-NMR has been used to aid in PEG-bound peptide synthesis [35, 37].

Infrared spectroscopy (IR) is of some limited utility for analyzing PEG's. The problem with this technique, as with proton NMR, is that the absorbing functional groups at the polymer terminals are necessarily present in low concentrations and thus give weak signals. Characteristic functional group absorbances can be detected for acids, esters, and aldehydes, and the disappearance of the PEG hydroxyl group can be followed [10, 13, 14, 30, 31, 38–40].

Classical qualitative organic analysis is useful in establishing endgroup functionality. For example, amines and acids can be titrated, amines give the nitrous acid test [41], aldehydes give the Schiff test [10] and form 2,4-dinitrophenylhydrazides [28], esters react with Nessler's reagent [10], reaction of PEG-Br produces bromide which can be titrated [42], and so on. These same tests can sometimes (e.g., amine titration) also be used quantitatively to determine the percent modification if it is assumed that the molecular weight has not changed during derivative preparation or if a quantitative method for the original functional group is also available. On the other hand, molecular weight can be determined in this fashion if it is assumed that all the endgroups are identical (i.e., that reaction is complete) and that the extent of branching is known; this "endgroup method" applied to the hydroxyl groups of PEG is the classical method for determining the molecular weight of PEG [40, 43]. Elemental analysis can also be used to determine the extent of PEG derivatization if the molecular weight is known [44].

All of the methods generally applied to determining molecular weights of polymers can be applied to PEG [45]. We will not attempt to review this large topic but will simply note that most of the workers in PEG synthesis have used either the endgroup method or size exclusion chromatography.

Radiolabeling is also a powerful technique for characterization, especially for derivatives which are not isolated or which are available only in small quantities. This approach can be used when the ligand to be attached to PEG is labeled [10] or when the PEG itself is labeled [46]. A slight variation on the former approach is illustrated by the reductive amination of PEG aldehyde with an amine and tritiated sodium cyanoborohydride [10].

II. PREPARATION OF SIMPLE DERIVATIVES

A. Ethers

Several PEG ethers of long-chain hydrocarbons are commercially available under various trade names (Triton N, Triton X, Tergitol, Brij, Sterox HJ, etc.) [47–49], primarily for use as nonionic surfactants. Synthesis is by ethylene oxide polymerization initiated by the appropriate alcohol [47–50]. For laboratory uses it is frequently preferable to prepare the desired ether from PEG by Williamson synthesis [14, 15, 51–54].

$$\text{PEG—OH} \xrightarrow[\text{2. R—Br}]{\text{1. } (CH_3)_3COK,\ C_6H_6} \text{PEG—O—R}$$

$$\text{PEG—OH} \xrightarrow[\text{2. } (CH_3)_2SO_4]{\text{1. NaOH, } CH_2Cl_2} \text{PEG—O—}CH_3$$

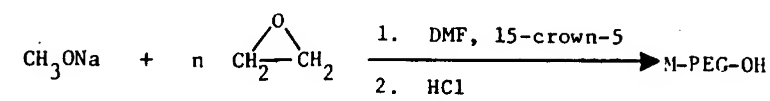
These reactions are straightforward, but product purification can be complicated for the larger alkyl groups. Toke purified his methyl and ethyl ethers of PEG 2000 by dissolving them in chloroform and washing repeatedly with water [52]. Sukata dissolved methyl ethers of PEG 1000 in benzene, washed with water, and chromatographed on alumina [15]. Similarly, Juri purified dimethyl ethers of PEG by chromatography on alumina [51]. Large alkyl derivatives require hydrophobic affinity chromatography (OctylSepharose) as the usual extraction and precipitation techniques will not remove trace amounts of alkyl bromide or alcohol [14]; a similar procedure is required for purifying fatty acid esters [14]. The products are readily characterized by chromatography, NMR spectroscopy, and elemental analysis. These procedures are will suited for preparing ^{14}C-derivatives, as the labeled acids are available and can be reduced to the corresponding alcohols with lithium aluminum hydride and converted to the halide or sulfonate [10, 54].

Several methods were used in the above reactions for preparing PEG alkoxides. Potentially superior methods are discussed in the section on acid preparations and below in the work of Cooke.

In addition to their use as surfactants, these simple ethers have found application in phase transfer catalysis [14, 55–58] and in the phase partitioning of red blood cells [29] and hydrophobic membrane proteins [59, 60].

The monomethyl ethers of PEG are commercially available and are frequently used as starting materials for the synthesis of other "mono" derivatives by substitution of the remaining hydroxyl group (refer to the section on Special Characteristics for a discussion of purity). Interestingly, Brunelle has shown that monoethers can be made selectively from hexaethylene glycol by proper choice of base and solvent [61].

Shalati has published a well-described laboratory polymerization of ethylene oxide to produce M-PEG having a molecular weight of approximately 1200 daltons [62]. Initiation of this polymerization with methoxide is hindered by the low solubility of methoxide in nonpolar solvents. Shalati overcame this problem by adding 15-crown-5 to chelate the sodium cation. Chelation also greatly enhanced the nucleophilicity of the alcoholate endgroup of the growing polymer. A further use of this M-PEG is described in the section on Polymer-Bound PEG. Related polymerizations are discussed in Ref. 63 and 64.



It should be noted that merely reducing reagent amounts to half the molar quantity of hydroxyl groups will not produce reaction of only one of the two hydroxyl groups of PEG to give a pure monoderivative; this comment applies in general to PEG derivatives, not just to ether syntheses. Applying the principles of probability leads to the following equations:

q^2 = fraction disubstituted

p^2 = fraction unsubstituted

$2pq$ = fraction monosubstituted

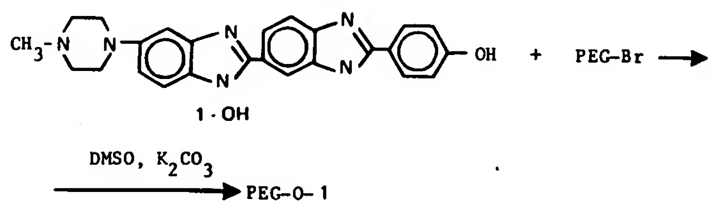
where p is the probability of a particular hydroxyl group not being substituted and q is its probability of being substituted. Thus, if 50% of the hydroxyl groups are substituted, 50% of the molecules will be monosubstituted, 25% will be disubstituted, and 25% will not be substituted; obviously this product mixture is quite different from the pure monoderivative one might naively have expected. This derivation is based on the generally valid assumption that the two ends of the PEG chain are independent.

An interesting variety of complex PEG ethers has been made. For example Mathias has made the vinyl ether by reacting the potassium salt of PEG with acetylene [65]:

$$\text{PEG—OH} \xrightarrow[\text{2. HC}\equiv\text{CH}]{\text{1. K}} \text{PEG—O—CH=CH}_2$$

This product can be polymerized to produce polyethylene with pendant PEG chains.

The preparation by Muller of PEG-dye conjugates provides another example of a complex ether [66]:

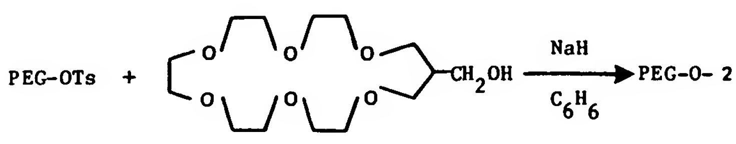


1·OH

PEG-O-1

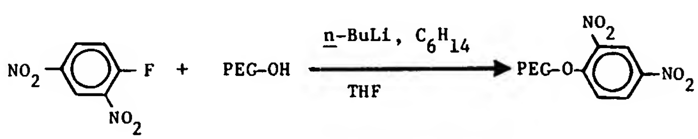
In this example the product is sufficiently larger than the reactants to permit purification by gel chromatography.

We have prepared the ether of PEG and crown ethers by reaction of the crown alkoxide with PEG tosylate [67]:



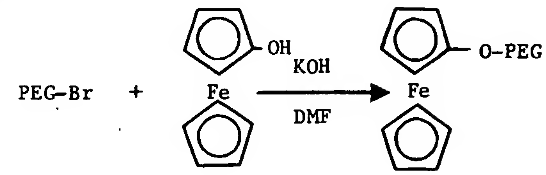
2·OH

Cooke has made 2,4-dinitrophenyl ethers of M-PEG 750 and PEG 68,000 by reaction of the PEG alkoxide with 2,4-dinitrofluorobenzene [68]:



Alkoxide formation by reaction with butyllithium has the advantage of being easily followed with 1,10-phenanthroline as an indicator for excess butyllithium. Purification was achieved by gel filtration chromatography. Toke has prepared similar phenoxy ethers by the Williamson route [52].

Akabori has synthesized PEG-ferrocene ethers by reacting the alkoxide of a ferrocene alcohol with PEG bromide [69]:



This derivative was examined along with several similar compounds for use in metal extractions.

Selve has prepared oligoethyleneoxide perfluoroalkyl ethers for use as blood substitutes [70]. Williamson ether synthesis gave much unwanted diether, as did polymerization of ethylene oxide with perfluoroalcohols. To obtain monoethers having the desired surfactant properties, Selve devised an interesting new approach based on the ability to prepare selectively the mono (tris-dimethylamino)phosphonium salts of tetra-, penta-, and hexaethylene glycols:

$$HO(CH_2CH_2O)_6H \xrightarrow[KPF_6]{PZ_3,\ CCl_4} HO(CH_2CH_2O)_6PZ_3^{+}PF_6^{-}$$

$$\xrightarrow{F_3C(CF_2)_6CH_2O^{-}Na^{+}} HO(CH_2CH_2O)_6CH_2(CF_2)_6CF_3$$

where $Z = N(CH_3)_2$. Larger glycol ethers could also be prepared, although in poorer yield, by reacting tetrahydropyran-protected glycol salts with the alkoxide of the oligoethyleneoxide ether prepared above.

B. Esters

Esters of PEG have many uses including applications in phase partitioning [71–75], drug attachment [11, 30, 76], detergency [77], peptide synthesis [44, 78–80], enzyme immobilization [81], and protection of the PEG hydroxyl group [82]. Several standard methods for ester synthesis have been used. For example, Johansson [74], Mutter [79], Glass [80], and Okamoto [82] have reacted PEG with acid chlorides:

$$\text{PEG—OH} + CH_3(CH_2)_{14}COCl \xrightarrow[\text{toluene}]{Et_3N} \text{PEG—}O_2C(CH_2)_{14}CH_3$$

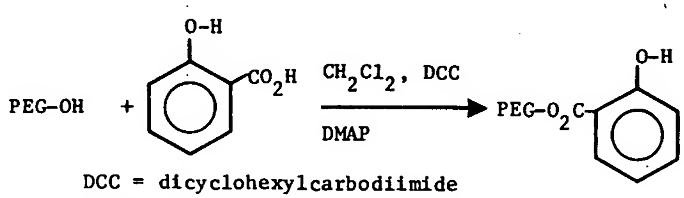
Mutter removed HCl from his reaction mixture with a stream of nitrogen [79] and Johansson used the usual approach of adding an amine. Interestingly, Okamoto and Glass did not remove HCl from their reactions. In view of the ease with which acid can cleave PEG chains [10, 83, 162], the processes having HCl removal would seem to be preferable.

The acid chloride can also be generated in the reaction mixture and used without isolation by use of oxalyl chloride [10]:

$$\text{PEG—OH} + CH_3(CH_2)_{14}CO_2H \xrightarrow[Et_3N,\ \text{toluene}]{ClCOCOCl}$$

$$\text{PEG—}O_2C(CH_2)_{14}CH_3$$

PEG can also be reacted directly with carboxylic acids using various catalysts such as carbonyl diimidazole [15, 66], cyclohexylcarbodiimide [30, 91], tosyl chloride and imidazole [66], and *p*-toluenesulfonic acid [81]:



DCC = dicyclohexylcarbodiimide

DMAP = 4-dimethylaminopyridine

Interestingly, Muller [66] used no solvent but ran his reaction in a melt of PEG. This, also, is the commercial route to PEG diesters.

Esters can also be made by ethylene oxide polymerization with a carboxylic acid salt as catalyst, but transesterification reduces the effectiveness of the preparation [50].

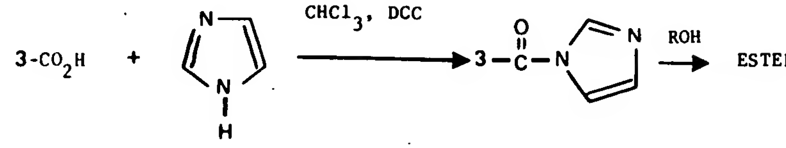
Several workers have prepared PEG carboxylic acids which were subsequently esterified [11, 30, 85]. The acid can be reacted in a single step or an active form prepared and isolated:

$$\text{PEG—}O_2CCH_2CH_2CO_2H + \text{atropine—OH} \xrightarrow[HOBt]{CH_2Cl_2,\ DCC}$$

$$3\text{—}CO_2H$$

$$\text{PEG—}O_2CCH_2CH_2CO_2\text{—atropine}$$

where HOBt = 1-hydroxybenzotriazole.



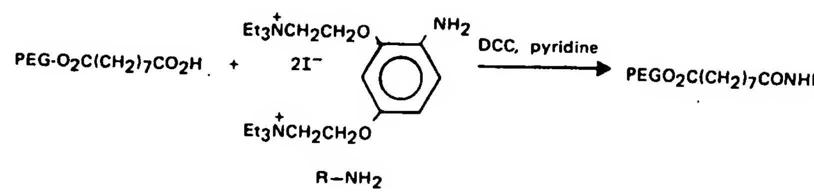
Related active esters for protein attachment are described in the section on protein conjugates.

Several complex esters are described in the section on peptide synthesis.

C. Amides

In addition to the active imidazole amides discussed in the preceding section, several other amides have been prepared, although there has not been quite the interest in these compounds as in the esters.

Johansson [85], Royer [12], Zalipsky [30], and Buckmann [86] have all prepared PEG amides using carbodiimides as coupling agents:



$$\text{MPEG—}O_2CCH_2CH_2CO_2H + C_6H_5\text{—}CH_2CH(CH_3)NH_2$$

$$\xrightarrow[HOBt]{DCC,\ CH_2Cl_2} MPEGO_2CCH_2CH_2CONH(CH_3)CHCH_2C_6H_5$$

$$PEG{-}O_2CCH_2CH_2CO_2H + H_2NCH_2CH_2{-}NAD^+ \xrightarrow[H_2O]{EDC} PEG{-}O_2CCH_2CH_2CONHCH_2CH_2{-}NAD^+$$

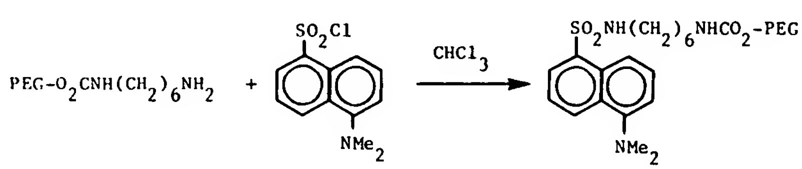
It is interesting to examine the differences in these four preparations. The Johansson [85] reaction was straightforward with dicyclohexylcarbodiimide (DCC) in pyridine. However, Zalipsky [30] found that his reaction failed with DCC in methylene chloride unless 1-hydroxybenzotriazole (HOBt) was added (an intermediate ester is formed with the PEG acid). Finally, Royer [12] and Buckmann [86] carried out their reactions in water using the water-soluble carbodiimide 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC).

The Johansson product was used in affinity partitioning of membrane fragments, while the Buckmann amide was used as a membrane impermeable coenzyme. Royer prepared his amide for use in peptide synthesis (below). The Zalipsky reaction was used for drug attachment.

The imidazole and benzotriazole active amides prepared by Ferruti and described in the previous section for ester synthesis are also well suited for preparing other amides [11]; amines, of course, are more nucleophilic than alcohols, and Ferruti has shown that reaction with amines is more likely to produce complete reaction with his active derivatives.

Hubert and colleagues have prepared PEG amides of the steroid estradiol, and have used this material for affinity partitioning of an enzyme which binds this steriod [87–89]. PEG amine and an acidic derivative of the steroid were used.

Okamoto has prepared fluorescent PEG dansyl sulfonamides from reaction of 5-dimethylamino-1-napthalenesulphonyl chloride (dansyl chloride) and PEG amines [18]:



This fluorescent derivative can be used to follow kinetics of polymer reactions and also to quantify the amine content of PEG amines.

D. Amines

PEG amines are important as intermediates in the synthesis of other derivatives, and several PEG amines are themselves useful in direct application. Consequently, several routes for their preparation have been explored.

Buckmann and Johansson have described two direct syntheses for primary PEG amines, the most useful form for subsequent derivatization [42, 85]:

$$PEG{-}Br \xrightarrow{EtOH,\ NH_3} PEG{-}NH_2$$

$$PEG{-}Br + H_2N(CH_2)_6NH_2 \xrightarrow{EtOH} PEG{-}NH(CH_2)_6NH_2$$

The first method requires use of a glass autoclave and handling of gaseous ammonia. Otherwise it is very direct and produces essentially 100% substitution. The second method is easily applied, and although a primary and secondary amine are produced, the primary is much more reactive. Since the hexamethylenediamine is difunctional, there is the possibility in this reaction of interconnecting PEG chains. Johansson has found this to occur if the molar ratio of bromide to amine is 0.19 but not if it is 0.05. Flanagan has used ethylenediamine in a similar fashion [90].

A direct route to oligo(oxyethylene) diamines is provided by the method of Kern [91]. Presumably this approach will also be effective for PEG's.

$$PEG{-}OTs + KOCH_2CH_2NH_2 \xrightarrow[C_6H_6]{(CH_3)_3COH} PEG{-}OCH_2CH_2NH_2$$

Mutter [44], Geckeler [39], and Ciuffarin [92] have applied the classic phthalimide-hydrazine procedure to the preparation of PEG amine:

$$MPEG{-}OTs \xrightarrow[H_2N{-}NH_2,\ EtOH]{K^+phthalimide,\ DMF} MPEG{-}NH_2$$

There are several steps to this procedure, but it has been used with great success in the many examples of peptide synthesis by Mutter and his colleagues.

PEG amine can also be prepared by reductive amination of PEG aldehyde with ammonium acetate [10]:

$$PEG—OCH_2CHO + NH_4OAc \xrightarrow[NaCNBH_3]{CH_3OH} PEG—NH_2$$

We have found this method to give essentially complete substitution with no chain cleavage.

Zalipsky has described an effective three-step synthesis of PEG amine which gives an 80% yield of completely aminated product (as shown by elemental analysis and titration) [30].

$$MPEG—Cl \xrightarrow[2.\ H_2,\ Pd/C,\ EtOH]{1.\ NaN_3,\ DMF} MPEG—NH_2$$

A fairly complicated route to the primary amine is provided by reaction with hexamethylenediisocynate to produce a monoisocyanate which is then transformed into the amine [18]:

$$MPEG—OH + OCN(CH_2)_6NCO \xrightarrow[Bu_2SnCl_2]{toluene}$$

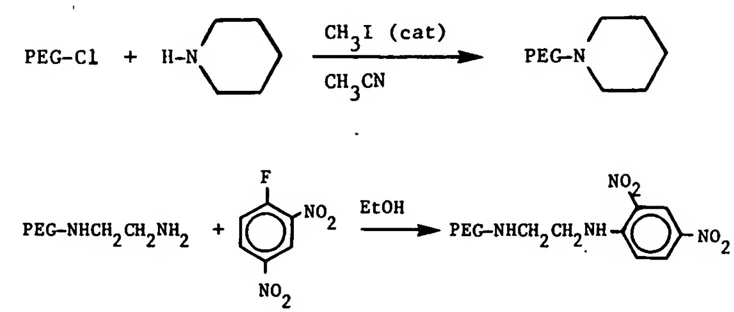
$$MPEG—O_2CNH(CH_2)_6NCO \xrightarrow[2.\ HCl]{1.\ (CH_3)_3COH}$$

$$MPEG—O_2CNH(CH_2)_6\text{-}NH_2$$

In this work Okamoto showed that the amine content of PEG amines can be quickly determined by the conversion to the fluorescent sulfonamide by reaction with dansyl chloride. This analysis shows that the isocyanate route to the amine gives fairly low degrees of conversion, for example, PEG 8800 had only 0.31 amino groups per chain. A very interesting aspect of this work is that the reaction rate of the amine with sulfonyl chlorides is shown to be independent of the degree of polymerization.

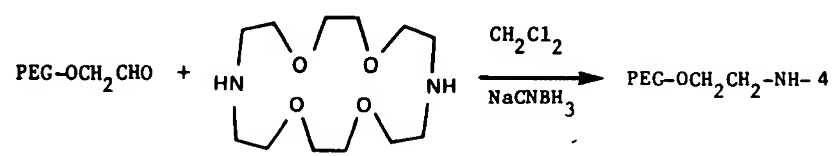
Several preparations of secondary and tertiary PEG amines have been published. Suzaki [93] and Szabo [52] have carried out displacements on PEG tosylate and chloride with amines,

while Flanagan [90] has adopted the approach of using PEG amine as the nucleophile:



Note that the Flanagan amine (used in phase partitioning) corresponds to the previously described dinitrophenyl ether prepared by Cooke [68] (for use as a structurally defined antigen).

We have made PEG-crown ether derivatives for use in phase-transfer catalysis by reductive amination of PEG aldehyde with crown amines [10]:



4 · NH

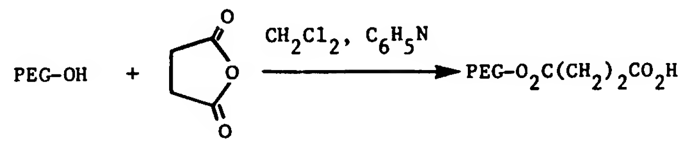
Johansson has prepared trimethylamino and triethylamino PEG salts for use in phase partitioning [42, 85]:

$$PEG—Br + (CH_3)_3N \xrightarrow{EtOH} PEG—N(CH_3)_3^{+}Br^{-}$$

Both of these products are useful in affinity phase partitioning, and the first is also of general utility for preparing charged PEG phases for phase partitioning.

E. Acids

As noted in the section on esters, several groups have used the reaction of PEG with succinic anhydride to prepare a PEG carboxylic acid [11, 30, 42, 46, 94]:



The reaction of PEG and succinic anhydride is rather slow (5 h at 150°C), but as Zalipsky has shown [30], the reaction is effectively catalyzed by triethylamine and dimethylaminopyridine so that complete reaction is obtained in 6 h at room temperature. Another approach is that of Boccu [95] and Buckmann [42] who showed that reaction of PEG amine and succinic anhydride is a quick route to the acid. Also, Boccu noted that the amide linkage is more stable under biological conditions than the ester linkage.

The other commonly used route to PEG carboxylate is via the alkoxide and a carboxylic acid activated in the alpha position [12, 13, 27, 42, 94]:

$$\text{PEG—OH} + \text{Na/naphthalene} + \text{Br—CH}_2\text{CO}_2\text{Et} \xrightarrow[\text{2. OH}^-]{\text{1. THF}} \text{PEG—O—CH}_2\text{CO}_2\text{H}$$

Sodium naphthalide is an especially useful reagent for preparing the alkoxide of PEG as the reaction is fast at room temperature and is also self-indicating; the reagent is simply added until its green color persists. The Geckeler derivatives [94] were used in peptide synthesis while the Buckmann derivative [42] was used in phase partitioning.

Johansson has also prepared PEG carboxylic acids by two other interesting routes. The first involves direct oxidation of PEG with permanganate [96]:

$$\text{PEG—OH} + \text{KMnO}_4 \xrightarrow[\text{H}_2\text{O}]{\text{NaOH}} \text{PEG—O—CH}_2\text{CO}_2\text{H}$$

Unfortunately, this route gives appreciable amounts of chain cleavage. The second reaction involves formation of an ester linkage with azelaic acid [85]:

$$\text{PEG—OH} + \text{HO}_2\text{C—(CH}_2)_7\text{—CO}_2\text{H} \xrightarrow[\text{DCCI}]{\text{C}_6\text{H}_5\text{N}}$$

$$\text{PEG—O}_2\text{C—(CH}_2)_7\text{—CO}_2\text{H}$$

This acid is useful in affinity phase partitioning as the hydrocarbon spacer moves the acid functionality some distance from the PEG.

Boccu has prepared a PEG carboxylic acid in two steps by oxidizing PEG to the aldehyde with manganese dioxide followed by further oxidation with hydrogen peroxide [97].

Purification of low molecular weight acids is readily achieved by extraction into chloroform at pH below seven and back into water at pH above seven. Anion-exchange chromatography can also be used.

F. Aldehydes

PEG aldehyde is potentially useful for coupling to amines by reductive amination [10, 98]. Three preparations of the aldehyde have been published. In the first, Royer reacts PEG with manganese dioxide [98]:

$$\text{PEG—OH} + \text{MnO}_2 \xrightarrow{\text{CH}_2\text{Cl}_2} \text{PEG—O—CH}_2\text{—CHO}$$

The aldehyde is not isolated or characterized in this procedure, but is only assumed to be present. Oxidation is followed immediately by coupling to proteins by reductive amination with sodium borohydride. Unfortunately, few details are given in the patent describing this work. Boccu has also used this same route to the aldehyde as part of a two-step synthesis of the PEG carboxylic acid. In this preparation methylene chloride was used as the solvent and reaction was continued overnight at room temperature [95].

We have prepared PEG aldehydes by two routes [10]. In the first, PEG alkoxide is reacted with the diethyl acetal of bromoacetaldehyde and the acetal decomposed to the aldehyde by reaction with dilute acid:

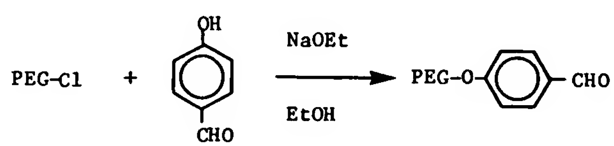
$$\text{PEG—OH} + \text{Br—CH}_2\text{CH(OEt)}_2 \xrightarrow[\text{2. HCl}]{\text{1. (CH}_3)_3\text{COK, C}_6\text{H}_6} \text{PEG—O—CH}_2\text{—CHO}$$

In the second, more direct process, PEG is oxidized by a mixture of dimethylsulfoxide and acetic anhydride:

$$\text{PEG—OH} + CH_3SOCH_3 + (CH_3CO)_2O \longrightarrow \text{PEG—O—}CH_2\text{—CHO}$$

The products were characterized qualitatively by Schiff test and quantitatively by reduction with tritiated sodium borohydride. The second process gives the better yield and is easier to apply. Subsequent to the above published work, we have found that traces of DMSO remaining in the aldehyde after precipitation can be removed by chromatography on LH-20. Our attempts to prepare the aldehyde with the usual pyridine chlorochromate reagent failed because of difficulty in removing metal impurities from the final product.

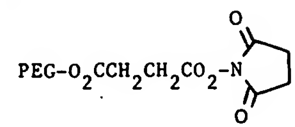
Bayer has prepared an aldehyde-terminated PEG by reacting PEG chloride with the phenoxide of 4-hydroxy benzaldehyde [28]:

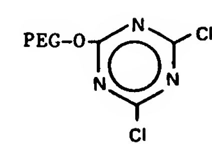


The product was characterized by preparing the 2,4-dinitrophenylhydrazone and determining the percent nitrogen in the product; a 91% conversion was noted. In addition, the ^{13}C-NMR spectrum of this derivative has also been described [32].

G. Electrophilic Derivatives

As the previous sections have illustrated, a major route to PEG derivatives is by nucleophilic substitution on reactive, electrophilic PEG's such as the chloride, bromide, tosylate, mesylate, or one of the active forms of PEG carboxylic acid. In this section these electrophilic derivatives are considered as a group, and methods are given for preparing and using those derivatives not yet described. These new derivatives include the chlorocarbonate, isocyanate, epoxide, and more complex derivatives such as the succinimidyl succinate and the cyanuric chloride derivatives:





PEG chloride and bromide have been prepared several times by reaction with thionyl chloride or bromide in toluene [30, 42, 99]:

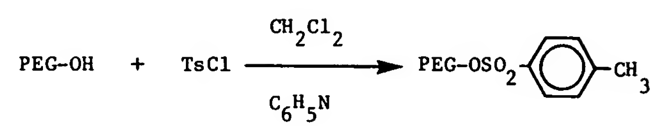
$$\text{PEG—OH} + SOBr_2 \xrightarrow[Et_3N]{C_6H_5\text{—}CH_3} \text{PEG—Br}$$

Johansson notes [99] that viscosity and gel chromatography indicate no change in molecular weight upon preparation of these derivatives. Our HPLC studies are in agreement with this conclusion [10]. Also, the bromide appears to be quite stable, as the sample we examined remained unchanged for at least 1 year.

Toke [52] and Bayer [28] have noted that the chloride preparation can be simplified by omitting solvent and simply running the reaction in an excess of thionyl chloride, which is subsequently removed by distillation.

The halide derivatives can be readily characterized by elemental analysis, hydrolysis followed by silver nitrate titration, and by infrared spectroscopy.

Mutter [44] and Suzaki [93] have prepared the tosylate by the usual route with pyridine as a acid scavenger:



HPLC examination [10, 67] indicates that this procedure produces a reduction in PEG molecular weight of approximately 30%. Also, we and others [100] have found this compound to decompose slowly upon storage. A preparation without chain cleavage is provided by eliminating the pyridine and preparing the PEG alkoxide by reaction with sodium hydride [67]:

$$\text{PEG—OH} + \text{TsCl} \xrightarrow[C_6H_6]{\text{NaH}} \text{PEG—OTs}$$

This result indicates that pyridine hydrochloride may be the agent causing chain cleavage in the first tosylate preparation. That this may be the case is indicated by preparing the tosylate [38] and mesylate [14] without cleavage by use of triethylamine as acid scavenger:

$$\text{PEG—OH} + CH_3SO_2Cl \xrightarrow[Et_3N]{CH_2Cl_2} \text{PEG—OSO}_2CH_3$$

We have found the mesylate to be stable upon storage. This factor, coupled with its greater reactivity relative to bromide, makes the mesylate a useful active intermediate. We compared the reactivities of the bromide, mesylate, and tosylate toward hydrolysis and found their relative rates to be, respectively, 1.0, 4.4, and 5.5 [10].

The isocyanate is a reactive electrophilic derivative which can be reacted with amines or alcohols to yield ureas or urethanes [18, 30, 101]:

$$\text{PEG—OH} + \text{OCN—}(CH_2)_6\text{—NCO} \xrightarrow{C_6H_5\text{—}CH_3}$$

$$\text{PEG—O}_2\text{CNH—}(CH_2)_6\text{—NCO} \xrightarrow{\text{ROH}}$$

$$\text{PEG—O}_2\text{CNH—}(CH_2)_6\text{—NHCO}_2\text{—R}$$

Zalipsky [30] found dibutyltin dilaurate to be an effective catalyst for the coupling reaction. Also, he noted that PEG isocyanate decomposes upon standing and thus should be freshly prepared before use. The urethane linkage appears to be useful as a labile linkage for controlled release of drugs [30].

Similarly, PEG chlorocarbonate has been prepared and reacted with an amine to make a urethane by Takerkart [102]:

$$\text{PEG—OH} + COCl_2 \xrightarrow{C_6H_5\text{—}CH_3} \text{PEG—O}_2\text{C—Cl} \xrightarrow{\text{R—NH}_2}$$

$$\text{PEG—O}_2\text{CNH—R}$$

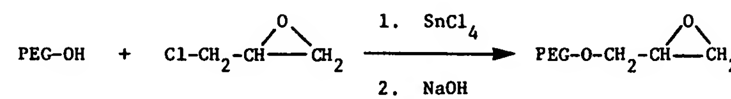
The attached *p*-aminobenzaldehyde is a trypsin inhibitor. Takerkart used this material in the affinity phase partitioning of trypsin.

The chlorocarbonate is also reactive toward other nucleophiles. Reaction with alcohols gives carbonates [103–106]. Nuzzo used this active derivative for preparing phosphine derivatives for binding transition metal catalysts [107]:

$$\text{PEG—O}_2\text{C—Cl} + HN(CH_2CH_2PPh_2)_2 \longrightarrow$$

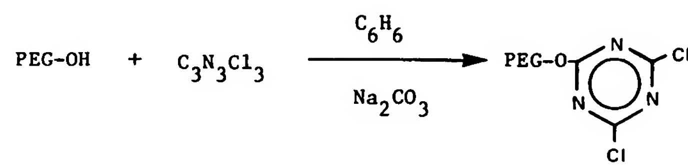
$$\text{PEG—O}_2\text{C—N}(CH_2CH_2PPh_2)_2$$

Pitha has prepared PEG epoxides by reaction of PEG with epichlorohydrin followed by reaction with sodium hydroxide [108]:



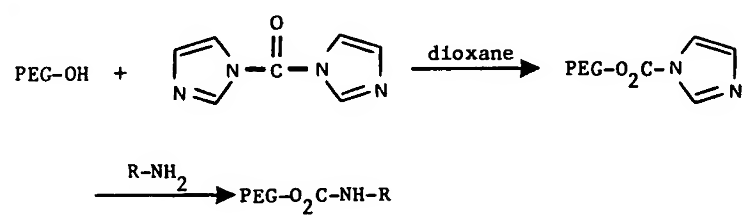
This derivative is sufficiently electrophilic to react with both primary and secondary hydroxyl groups of polysaccharides (see Polymer-Bound PEG).

There has been a great deal of interest in electrophilic PEG's for coupling to free amino groups (lysines) of proteins. These same derivatives are potentially useful for reaction with other nucleophiles although they have not been used in this fashion as yet. The most used derivative for protein attachment has been prepared by reacting PEG with cyanuric chloride [19]:

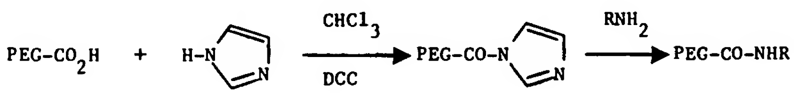


The first of the two chlorines on this derivative is substituted by protein amino groups in about a day. The second chlorine is much less reactive, although there does appear to be some reaction at this site [109].

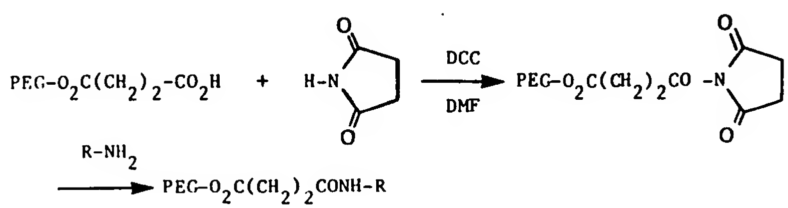
Beauchamp has reacted PEG with carbonyldiimidazole to prepare the following electrophilic PEG urethane [110]:



This intermediate is said to give less deactivation upon reaction with proteins (to form a urethane) than the above cyanuric chloride derivative. The Beauchamp urethane is similar to the amide prepared by Ferruti [11] for ester and amide syntheses:



Another electrophilic PEG for protein attachment has been prepared by Abuchowski [111]:



This derivative is said to be more reactive and less deactivating than the cyanuric chloride derivative.

H. Miscellaneous Derivatives

There are a few derivatives for which only a single report exists. Those discussed in this section include a urethane, an isourethane, a sulfonate, and a tertiary alcohol.

We earlier reviewed work in which urethanes were made by reaction of PEG chlorocarbonate with amines and PEG isocyanate with alcohols. An alternative route to urethanes, which was not covered, is reaction of PEG with an alkyl isocyanate. Mutter has used this approach in coupling PEG to various isocyanates [79]:

$$\text{PEG—OH} + \text{Cl—}(CH_2)_6\text{—NCO} \xrightarrow{CH_2Cl_2} \text{PEG—}O_2\text{CNH—}(CH_2)_6\text{—Cl}$$

This procedure is especially effective for introducing complex groups having a second reactive site for attaching a growing peptide chain.

Mathias has prepared isourethanes of oligomeric ethylene glycols by reaction with carbodiimides [112]:

$$\text{PEG—OH} + \text{R—N=C=N—R} \longrightarrow \text{R—NH—}\overset{\overset{\text{N—R}}{\|}}{\text{C}}\text{—O—PEG}$$

5

where R = $(CH_3)_2CH$—. The isourethanes are useful in preparing interesting polymers containing both sulfur and oxygen:

$$\mathbf{5} + HS(CH_2)_3SH \xrightarrow{KF} \left[S(CH_2)_3\text{—S—}(CH_2CH_2O)_xCH_2CH_2 \right]_y$$

As noted above, carboxymethylated PEG is useful for forming polymer-phase systems containing a negatively charged PEG. Similarly, PEG sulfonate can also be used in this fashion [99]:

$$\text{PEG—Br} + Na_2SO_3 \xrightarrow[H_2O]{EtOH} \text{PEG—}SO_3^-\,Na^+$$

Viscosity and gel chromatography indicate this product is formed without chain cleavage.

PEG is itself an alcohol, so there has been little need to make new PEG alcohols; however, Anzinger needed a PEG tertiary alcohol as an acid labile protecting group for peptide synthesis, and prepared the following derivative [31]:

$$\text{M—PEG—OH} + BrCH_2CO_2Et \xrightarrow{NaNaph} \text{M—PEG—O—}CH_2CO_2Et \xrightarrow{CH_3MgI} \text{M—PEG—O—}CH_2C(CH_3)_2OH$$

III. SYNTHESIS OF COMPLEX DERIVATIVES OF PEG

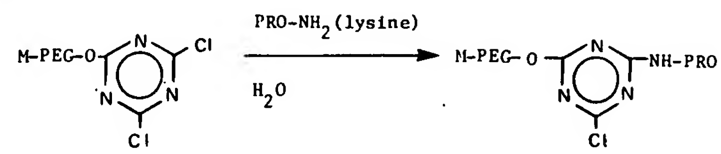
The topics covered in this section have been chosen abritrarily to illustrate the broad range of derivatives that have been prepared and to give an indication of the many applications of PEG derivatives. As was the case in the previous section, polymerization processes are not considered; all the derivatives examined have been prepared from PEG or one of its commercially available alkyl ethers.

A. Protein Conjugates

Synthesis of protein conjugates is an active area of research because of the fascinating modifications of protein properties which result. For example, administration of PEG conjugates of protein antigens to test animals results in immunosuppression of the animals immune system such that future exposure to antigens does not provoke an allergenic response [113–116]. Attaching PEG to enzymes produces a decrease in enzyme immunogenicity and antigenicity and an increase in serum lifetime with the result that PEG-enzymes can be used as drugs [97, 110, 116–122]. The increased serum lifetime of PEG-proteins is also used to advantage in modifying hemoglobin to be used as a blood substitute [123]. Binding a protein to PEG can also facilitate further modification of the protein [80]. Another area under active investigation is binding antibodies to PEG for use in affinity phase partitioning of cells and membrane fragments [124, 125]. Mattiasson and his co-workers have used affinity phase partitioning with PEG-bound lectins and antibodies to provide a novel approach to immunoassays for cells and molecules [126–128]. In a novel use of PEG-proteins, Anzai has attached the nonylphenoxy ether of PEG to poly(methyl glutamate) by transesterification and has used the product for metal cation extraction through a liquid membrane [129].

The basic approach to preparing protein-PEG conjugates is to synthesize an "activated" PEG having a reactive functional group that can be readily coupled with some functional group on the protein. With two exceptions, the works cited use as protein functional group the amino group of lysine subunits.

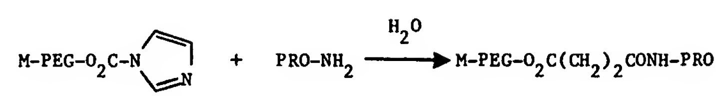
The most frequently applied route is that of Abuchowski and Davis in which the PEG-cyanuric chloride derivative (refer to Electrophilic Derivatives) is reacted with the protein [18, 19, 114, 119, 123, 124, 127, 128]:



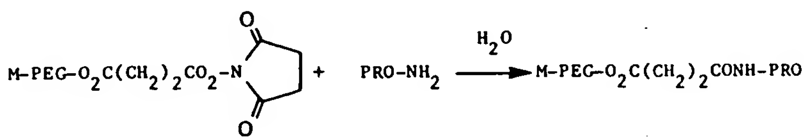
Analysis of the protein-PEG conjugate requires measurement of protein concentration of the purified material (ultrafiltration or gel chromatography). Abuchowski suggests the biuret method for this determination as he has shown this method, unlike the much used Lowry determination, to be unaffected by attached PEG [19]; it is essential for use of the biuret method that all free PEG be removed during the purification step. Brooks has used I-125 labeled protein and fluorescence in place of the biuret procedure for protein determination [125]. The final step is to determine the number of lysine groups substituted during reaction; this is done by further reaction with trinitrobenzenesulfonic acid and measurement of the resulting ultraviolet absorption of the newly introduced group [130]. To achieve reliable results, solutions of trinitrobenzenesulfonic acid must be prepared shortly before use.

A prime practical consideration when using PEG-protein conjugates is to determine the optimum degree of substitution and PEG molecular weight needed to give the desired effect (on immunogenicity or partitioning, for example) without producing excessive protein deactivation. There appear to be no general rules in this regard, as effects vary greatly from system to system. Consequently, it is necessary to determine the effects of PEG molecular weight and degree of substitution in each case.

The cyanuric chloride method is not ideal in that loss of protein activity can be significant [110, 111], particularly with enzymes having active sulfhydryl groups with which the activated PEG can react [95]. Boccu has also noted that the cyanuric chloride activated PEG produces some cross-linking because of the additional reactive chlorine [109]. Consequently, other methods for protein-PEG union have been investigated. In one alternative route Beauchamp reacts PEG with carbonyldiimidazole to produce an activated derivative subject to nucleophilic attack by lysine amino groups [110]:



A second approach utilizing a succinimidyl "active ester" has been examined by three groups [42, 95, 97, 111]:



An early version of this approach is the synthesis by Royer [131] of the bis(succinimidyl succinate) of ethylene glycol. The Beauchamp approach gives superoxide dismutase having 100% of lysine groups substituted that retains 95% of its native activity; this compares with 51% retention of activity with the cyanuric chloride method [110]. The second method gives asparaginase with 52% retention of original activity, whereas the cyanuric chloride approach leads to almost complete deactivation of this enzyme [111]. Veronese used the succinimidyl preparation to attach PEG to superoxide dismutase and found little deactivation; for example, attaching six PEG's gave a product retaining 80% of the activity of the native enzyme [97]. These workers also examined other enzymes [95].

Another activated PEG has been prepared from the PEG succinate by Lee by reacting the acid with isobutylchloroformate [113]:

$$\text{M—PEG—}O_2C(CH_2)_2CO_2H + RO_2CCl \longrightarrow$$

$$\text{M—PEG—}O_2C(CH_2)_2CO_2\text{—}CO_2\text{—R} \xrightarrow{\text{PRO—}NH_2}$$

$$\text{M—PEG—}O_2C(CH_2)_2\text{—CONH—PRO}$$

The resulting mixed anhydride is rapidly substituted by lysine amino groups.

King has prepared an active dithiocarbonate PEG which readily attaches to proteins [115]:

$$\text{M—PEG—}O^-Na^+ \xrightarrow[\text{2. } ClCH_2CONH_2 \quad DMSO,\ C_6H_6]{\text{1. } CS_2,\ C_6H_6}$$

$$\text{M—PEG—OSC—}SCH_2CO\text{—}NH_2 \xrightarrow{\text{PRO—}NH_2}$$

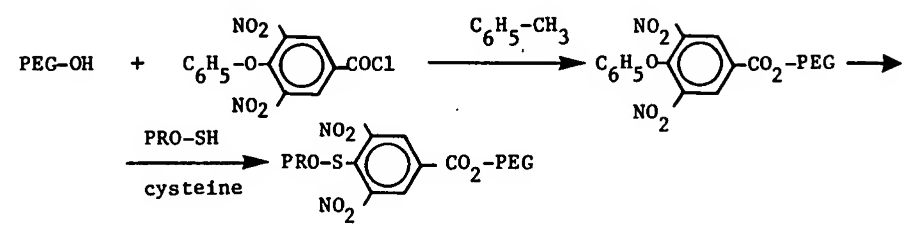
$$\text{M—PEG—OSC—NH—PRO}$$

A direct route to PEG-protein conjugates is provided by reductive amination of PEG aldehyde and lysine amino groups [10, 98, 132]:

$$\text{PEG—O—}CH_2\text{—CHO} + \text{PRO—}NH_2 \xrightarrow[H_2O,\ pH\ 8]{NaCNBH_3}$$

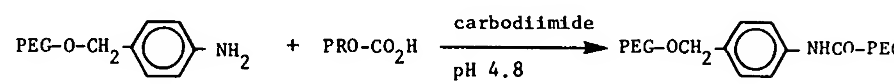
$$\text{PEG—O—}CH_2\text{—}CH_2NH\text{—PRO}$$

Glass has developed a novel method for attaching PEG to proteins by attack on cysteine sulfhydryl groups rather than the usual attack on lysines [80]:



An advantage of this route is that the free protein can be regained easily by reaction with 2-mercaptoethanol, thus facilitating preparation of modified proteins.

In a second work involving binding through a group other than lysine, Pollak has reacted a PEG amine with an enzyme (glucose 6-phosphate dehydrogenase) in the presence of a coupling agent [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride], presumably to yield amide linkages [146]:



The number of PEG's attached was not determined, but it was noted that 22% retention of enzyme activity was achieved.

The PEG-conjugates described to this point have all been covalently bound. In an interesting variation, Fukui has immobilized the enzyme yeast invertase in a PEG matrix [81]. The procedure is to make a thin film on transparent polyester film of PEG-dimethacrylate, enzyme, and initiator (benzoin ethyl ether) and to polymerize the dimethacrylate by illumination with

UV light. The result is a highly cross-linked PEG in which the enzyme is physically immobilized. The matrix remains permeable to substrate, and the bound enzyme retains 40–60% of its activity. Fukui used the material by cutting the film into small pieces which were added to reaction mixtures.

B. Peptide Synthesis

In the early 1970s, Bayer and Mutter introduced a method for peptide synthesis based on attaching a growing peptide chain to soluble PEG rather than to an insoluble matrix as had previously been done [78, 79, 84, 133]. There appear to be several advantages to this approach. In particular, the soluble polymer provides more precise control of chemistry and gives more rapid and complete reaction. Removal of products from excess reactants is readily achieved by the standard techniques of ultrafiltration, chromatography, or precipitation. PEG-peptide purity can be assessed directly at each step by use of either ^{13}C or proton (faster) NMR [35–37]. In addition to their use for peptide synthesis, the PEG-peptide conjugates, having enhanced solubility and crystallinity, are also useful in the study of peptide conformations by the usual spectroscopic and crystallographic techniques [35–37, 134, 135].

We will not attempt to review this area completely but rather will give some examples with the aim of illustrating difficult aspects of the approach and advantages resulting from the use of PEG. The basic approach as illustrated by the original papers is to attach the peptide to PEG by some functional group which can be cleaved selectively under conditions different from those required for removing amino-protecting groups from the end of the growing chain and sufficiently mild as not to attack the peptide amide linkages [78, 84]:

$$\text{M—PEG—Y + Z—NH—CHR—CO}_2\text{H} \xrightarrow{\text{couple}}$$

$$\text{M—PEG—X—CO—CHR—NH—Z} \xrightarrow{\text{deprotect}}$$

$$\text{M—PEG—X—COCHR—NH}_2 \xrightarrow[\text{ZNH—CHR'CO}_2\text{H}]{\text{couple}}$$

$$\text{M—PEG—X—COCHRNH—COCHR'NHZ}$$

Thus to prepare the above dipeptide mild and selective methods are required for removing Z and for cleaving X. The original

approach of Mutter and Bayer was to attach the PEG to the peptide by an ester linkage cleavable by 0.1*N* base and to use the *t*-butyloxycarbonyl (BOC) protecting group that is readily removed by mild acid:

$$\text{M—PEG—OH + ZNHCHRCO}_2\text{H} \xrightarrow{\text{1,1'-carbonyldiimidazole}}$$

$$\underset{\mathbf{6}}{\text{MPEGO}_2\text{CCHRNHZ}};\quad \mathbf{6} + \text{H}^+ \longrightarrow \text{MPEGO}_2\text{CCHRNH}_2$$

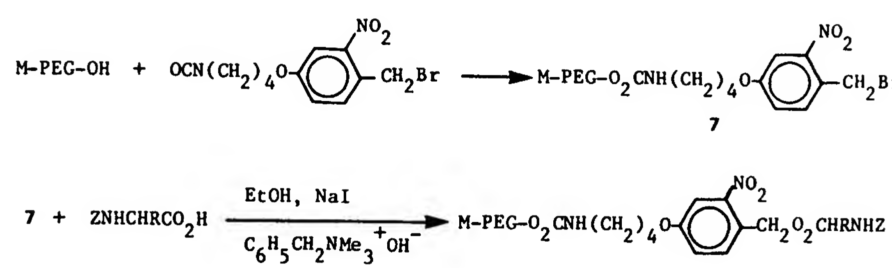
$$\xrightarrow{\text{ZNHCHR'CO}_2\text{H}} \text{MPEG—O}_2\text{CCHRNH—COCHR'NHZ}$$

$$\xrightarrow{\text{OH}^-} \text{M—PEG—OH + ZNHCHR'CO—NHCHRCO}_2\text{H}$$

where Z = *t*-butyloxycarbonyl. The ease of purifying PEG derivatives facilitates clean up at each stage.

The power of this approach has been demonstrated by the synthesis of an important decapeptide and by the synthesis of a segment of insulin [35, 136].

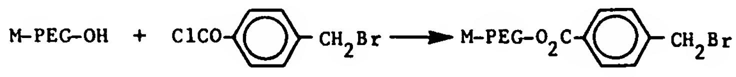
The base-catalyzed cleavage step produces some peptide racemization, and recent work has been directed toward obtaining PEG-peptide union by functional groups which are more readily cleaved [137]. Mutter has summarized much of this work in a recent paper detailing the preparation of some rather complex PEG derivatives permitting peptide attachment by active ester linkages [79]. Several urethanes were examined having terminal benzyl bromide moieties permitting formation of benzyl esters upon reaction with N-protected amino acids. Most, however, were not stable to the acid conditions required for removing the BOC group. Compound 7 was satisfactory, however:



The benzyl ester could be cleaved readily by dilute acid or by hydrogenation.

Certain alkyl urethanes and a PEG ester were also shown to be satisfactory:

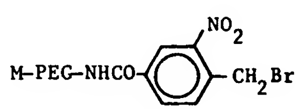
$$\text{M-PEG-OH} + \text{OCN}(\text{CH}_2)_6\text{-Cl} \longrightarrow \underset{\mathbf{8}}{\text{M-PEG-O}_2\text{CNH}(\text{CH}_2)_6\text{-Cl}} \xrightarrow{\text{as above}}$$



9

Compound 8 gave esters upon reaction with amino-protected amino acids which could be cleaved with mild base. Similarly, esters from 9 could be selectively cleaved by hydrogenation or by treatment with a mixture of hydrobromic and trifluoroacetic acids. In both cases the linkage to PEG was stable to these cleavage conditions.

In a final contribution from this paper, Mutter notes that peptides attached to compound 10 by an ester linkage can be cleaved by photolysis at 350 nm:



10

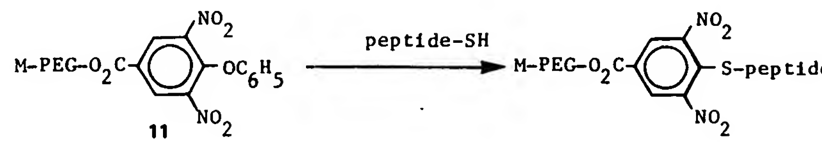
This photochemical method has also been examined by Pillai [138, 139], Bellof [140], and Tjoeng [141]. Tjoeng has also used hydrogenation for removal of peptides [142].

Royer has developed a variation of the liquid-phase method in which the peptide is attached to carboxymethyl-PEG-glycylmethionine [12]. Peptide linkages to this polymer are readily cleaved with cyanogen bromide. Another novel feature of Royer's approach is that the individual amino acids are blocked as ethyl esters which are selectively deblocked by carboxypeptidase Y. This enzymatic deblocking is especially attractive in that optical purity of the product is ensured; if the penultimate amino acid or the one at the terminus happens to have been racemized to the D form, the enzyme will not remove the protecting group.

An improved method for preparing the above benzyl derivatives has been introduced by Hemmasi who showed that better

results are achieved if the benzyl ester of the peptide is prepared first and then coupled to PEG [137].

An especially mild method of peptide removal is provided by the work of Glass with cysteine-containing peptides [80]:



11

There are two key aspects of this work. First, the dinitrophenolate linkage of 11 is selectively broken by thiol groups and is impervious to attack by amino and hydroxyl groups. Second, the sulfide bond joining the peptide to the dinitrobenzene group can be selectively cleaved by reaction with 2-mercaptoethanol.

In another refinement on selective removal of the PEG chain, Mutter has used a PEG tertiary alcohol (see above, Miscellaneous Derivatives) as an acid-labile carrier that can be attached to either the carboxyl or amino terminus of the growing peptide [31]:

$$\text{M—PEG—O—CH}_2\text{C(CH}_3)_2\text{OH} + \text{Cl—CO—F} \longrightarrow$$

$$\text{M—PEG—O—CH}_2\text{C(CH}_3)_2\text{—O}_2\text{C—F} \xrightarrow{\text{RNH}_2}$$

$$\text{M—PEG—O—CH}_2\text{C(CH}_3)_2\text{—O}_2\text{C—NHR}$$

$$\text{M—PEG—O—CH}_2\text{C(CH}_3)_2\text{OH} + \text{RCO}_2\text{H} \xrightarrow[\text{DMAP}]{\text{DCC}}$$

$$\text{M—PEG—O—CH}_2\text{C(CH}_3)_2\text{—O}_2\text{CR}$$

In the case of amino attachment, the PEG is joined to the peptide by a urethane linkage which is readily cleaved by mild acid. Similarly, the carboxyl attachment is through an acid-labile ester linkage.

All the work on peptide synthesis presented to this point has involved a growing peptide chain attached to a PEG carrier. Mutter has also presented an alternative method, little used to this point, in which the growing peptide chain is free, and added individual amino acids are bound to PEG by a linkage which is broken upon reaction with the amino end of the growing peptide [143]:

$$H_2NCHRCOQ + M{-}PEG{-}X{-}OCCHR'{-}NHZ \longrightarrow ZNHCHR'CO{-}NHCHRCOQ + PEG{-}X{-}H$$

The advantage of this approach is that excess added amino acid can be removed from the growing peptide by ultrafiltration. As in the preceding equations Z and Q represent suitable protecting groups, while X represents some linkage which can be cleaved without affecting the peptide amide linkages.

In an interesting variation on the PEG-bound approach to peptide synthesis, Colombo has attached the growing peptide to PEG by linkages which when cleaved give a peptide hydrazide useful in standard, solid-phase peptide synthesis [144]:

$$M{-}PEG{-}OCH_2C(CH_3)_2{-}O_2CNHNH_2 + RCO_2H \xrightarrow[HOBt]{DCC}$$

$$M{-}PEG{-}OCH_2C(CH_3)_2{-}O_2CNHNHCOR \xrightarrow[growth]{peptide}$$

$$M{-}PEG{-}OCH_2C(CH_3)_2{-}O_2CNHNHCOR' \xrightarrow{H^+}$$

$$R'CONHNH_2$$

where R = amino-protected peptide, R' = larger protected peptide.

PEG-bound peptides can also be used in solid-phase synthesis by using the liquid-phase chemistry to build peptides on PEG bound to polystyrene (PS) [145]. These supports are said to have advantages over both conventional solid- and liquid-phase syntheses since the peptide grows with favorable kinetics on the well-solvated PEG, yet the handling ease of solid-phase synthesis is retained. Also, varying the PEG to PS ratio permits control of the hydrophobic-hydrophilic balance of the solid polymer.

C. PEG-Bound Reagents and Catalysts

There has been much recent work on binding low molecular weight reagents and catalysts to solid polymeric supports to simplify separation and recovery of products and reagents. The PEG-peptide derivatives described in the previous section are simply a soluble variant on this theme, and bring to mind the possibility that advantages would result from attaching PEG to many of the reagents already attached to solid supports. Little has been done in this area as yet, but it should receive increasing attention in the future.

Carbodiimides are very important coupling agents for preparing esters, amides, and anhydrides. Mutter has prepared a PEG-substituted carbodiimide and used it in the synthesis of anhydrides from carboxylic acids [44]:

$$PEG{-}NH_2 \xrightarrow[2.\ TsCl,\ Et_3N]{1.\ i{-}Pr{-}NCO} PEG{-}N{=}C{=}N{-}C(CH_3)_2$$

The PEG-carbodiimide can be recovered and recycled several times.

Transition metal catalysts (e.g., for hydroformylation and hydrogenation) can also be readily dissolved in unusual solvents and recovered by using PEG-phosphine ligands [10, 107]:

$$M{-}PEG{-}O_2C{-}N(CH_2CH_2PPh_2)_2 \qquad M{-}PEG{-}PPh_2$$

$$(PEG{-}O)_2P{-}Ph$$

Similarly, Kula has bound the coenzyme NADH to PEG (see Amides) [86]. This material can then be retained in an enzyme reactor with an ultrafiltration membrane that permits products to escape.

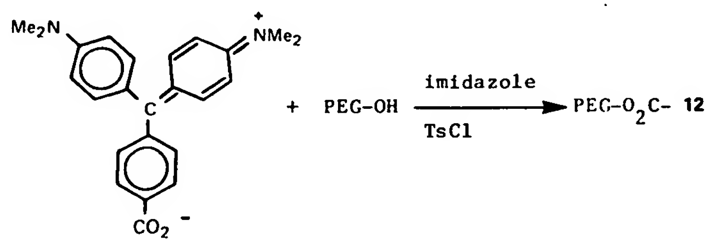
Benefits also result from binding enzymes to PEG and using them as catalysts. For example, Pollak has bound PEG to glucose 6-phosphate dehydrogenase and used this material in a PEG-dextran phase system to facilitate recovery of product and enzyme [146]. Similarly, we have attached acid phosphatase to PEG and used it to convert 2,4-dinitrophenylphosphate to 2,4-dinitrophenol in a PEG-dextran phase system [147]. In these phase systems the PEG-enzyme and the substrate can be partitioned to the PEG phase while the product partitions to the dextran phase.

In a related work, Mattiasson has shown that yeasts and their substrates can be partitioned to the dextran phase while the product partitions to the PEG phase [148]. In this case the catalyst (yeast) is not covalently bound, but the result is the same.

D. Dye Conjugates

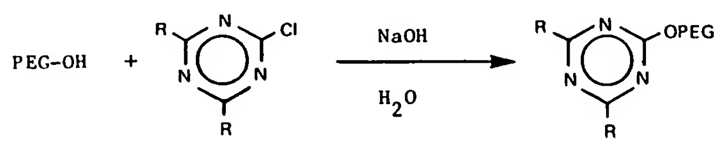
There has been much recent interest in PEG-bound dyes. These dye conjugates exhibit selective affinity in complexing with enzymes and nucleic acids and have been used in phase-partitioning purification of these materials. These purifications are of special interest because, unlike most laboratory purification methods, they may be easily scaled up and are thus well suited for industrial processes [149, 150].

Muller has established chromatography with solid-bound dyes as a standard method for purifying nucleic acids, and has recently used some of these same dyes bound to PEG for purifications by phase partitioning [66]. Two general routes for attachment are used, one giving a PEG ether (see Ethers) and the other giving a PEG ester:



$12-CO_2^-$

Johansson and Kula have examined many triazine dyes for their selectivity in complexing with enzymes, and have developed methods for attaching these dyes to PEG by taking advantage of the high susceptibility of triazine chlorides to nucleophilic attack [16, 17, 150]:



Johansson has shown lithium hydroxide to be superior to sodium hydroxide [17]. The products are readily purified by ion-exchange chromatography on DEAE-cellulose.

E. Drug Conjugates

The PEG-protein conjugates discussed earlier are of intense interest because the dramatic reduction of antigenicity and immunogenicity and the equally dramatic increase in serum lifetime greatly enhance the use of these materials as drugs (e.g., as antitumer agents and in immunotherapy for allergies). These same physiological properties can be imparted to nonprotein materials also. In addition, PEG is nontoxic [7–9] and, of course, has a wide range of solubilities. Thus PEG is highly attractive as a drug carrier.

Nonprotein drugs attached to PEG include procaine [103[, atropine [30, 104], various salicylates [30, 105], penicillin V [30], cannabidiol [106], amphetamine [30], quinidine [30], cinerubin A [151], and 4-isobutylphenyl-2-propionic acid [76].

The drugs have been attached to PEG through several functional groups already discussed. These include esters, amides, carbonates, and urethanes. For example, Cecchi has attached PEG to the antiinflammatory drug 4-isobutylphenyl-2-propionic acid by an ester linkage using carbonyldiimidazole as a coupling agent [76]:

$$RCO_2H \xrightarrow[\text{2. PEG—OH}]{\text{1. carbonyldiimidazole}} \text{PEG—}O_2C\text{—R}$$

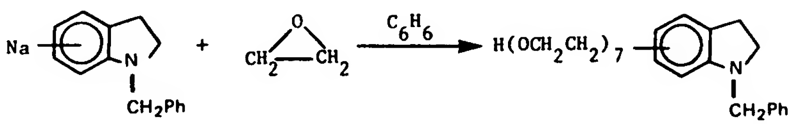
where $R = —CH(CH_3)—C_6H_5—CH(CH_3)_2$

Zalipsky has attached atropine, an anticholinergic compound, to PEG by means of a urethane linkage using dibutyltin dilaurate as a catalyst [30]:

$$\text{PEG—}O_2CNH(CH_2)_6NCO + \text{R—OH} \xrightarrow{C_6H_5CH_3}$$
$$\text{PEG—}O_2CNH(CH_2)_6NHCO_2\text{—R}$$

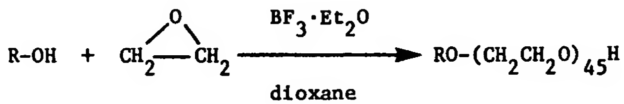
where R = atropine. Although it is not clear whether other functional groups provide controlled release of drugs from PEG, it does appear that this is the case for the urethane linkage [30].

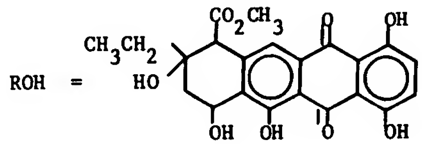
Weiner has prepared oligo(ethylene oxides) bound to various indolines by initiating ethylene oxide polymerization with sodium indolines [64]:



Removal of the benzyl group and subsequent modification gave PEG-bound indoleacetic acid and tryptamine.

As a final example we give Geckeler's polymerization of ethylene oxide by pyrromycinone (a cinerubin A antibiotic fragment) [151]:





An interesting aspect of this work is that the native antibiotic possesses a trisaccharide chain attached to pyrromycinone. Geckeler has replaced the trisaccharide with PEG and finds that the PEG derivative retains antibiotic activity.

F. Solid-Bound PEG

There is a great deal of interest, especially in industry, in attaching PEG to modify the properties of solid polymers such as cellulose, polystyrene, and silica. We will not attempt to review this area comprehensively but will give a few examples of the types of applications which are possible.

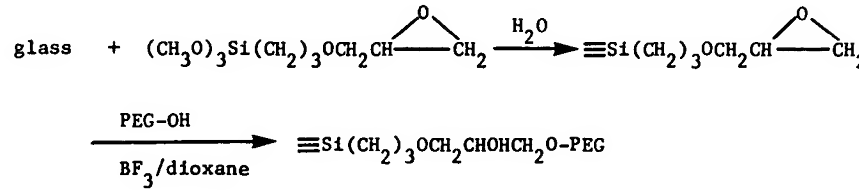
PEG bonded to silica provides an important type of chromatography support. The chemistry used is generally of two types. In the first, recently reviewed by Driscoll [152], the silica is treated with silicon tetrachloride and then with PEG:

$$\equiv Si\text{-}O\text{-}SiCl_3 + PEG\text{-}OH \longrightarrow \equiv Si\text{-}O\text{-}Si(OPEG)_3$$

Since one hydroxyl terminus of PEG remains unchanged, further modification (e.g., adding phthalic anhydride) can be done. The

Si—O—C linkage produced does, however, hydrolyze readily, so these supports cannot be used with aqueous samples.

The second approach is to treat the silica with a trialkyoxyorganosilane to form a water-stable Si—O—Si—C linkage; subsequent modification of the organic group provides the final product [10, 163]:



In a related work we have bound PEG to glass by reductive amination with PEG aldehyde of glass which has been treated with trimethoxyaminopropylsilane [10].

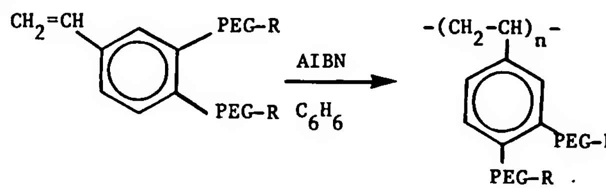
Polystyrenes (PS's) with PEG's or oligo(ethylene oxide)s attached have been actively investigated as phase transfer catalysts [57, 153–156], as polymeric emulsifiers [63], as supports for peptide synthesis [145], and as HPLC packings for separating metal cations [157]. The polymer-bound PEG can be made by reacting chloromethylated PS (a few percent cross-linked) with PEG alkoxide [63, 153, 155]. More complex derivatives with various groups (alkyl, quinolyl, naphthyl) attached to the polyether terminus have been prepared by substitution of alkoxides on PS-bound oligo(ethylene oxide) tosylate [63] and by polymerization of substituted styrene [156]:

$$PS\text{—}CH_2Cl + HO(CH_2CH_2O)_4H \xrightarrow[C_6H_5CH_3]{NaH}$$

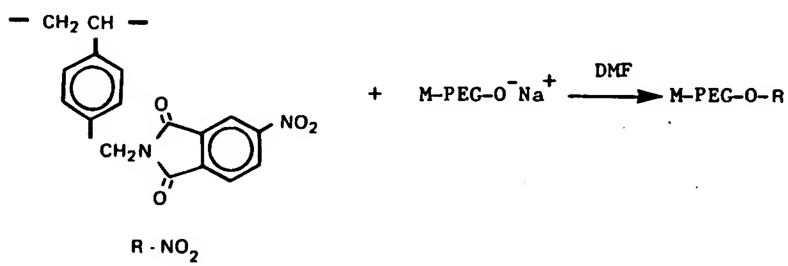
$$PS\text{—}CH_2O(CH_2CH_2O)_4H \xrightarrow[TsCl,\ C_6H_5N]{CH_2Cl_2}$$

$$PS\text{—}CH_2O(CH_2CH_2O)_4Ts \xrightarrow[EtOH]{RO^-K^+}$$

$$PS\text{—}CH_2O(CH_2CH_2O)_4R$$

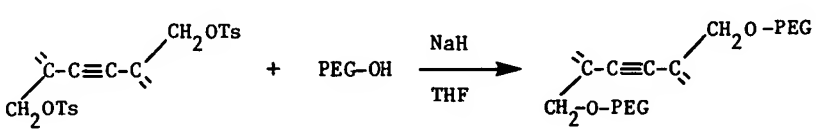


Shalati has developed an interesting method for grafting M-PEG onto polystyrene which has had 4-nitrophthalimidomethyl groups grafted previously [62]:



Quantitative grafting was obtained only in the presence of crown ether, which apparently functions by chelating the sodium cation. It is also noteworthy that the M-PEG used in this reaction was synthesized and reacted *in situ*.

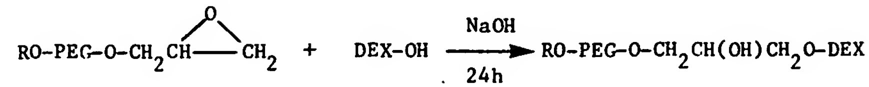
Kiji has grafted PEG onto polydiacetylene, also for use in phase transfer catalysis [158]:

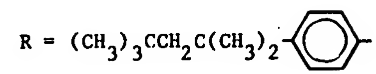


Polymeric emulsifiers can be made by grafting PEG onto poly(methyl methacrylate) by transesterification of the methacrylate with PEG alkoxide [159]. In a related work Hradil has bound PEG to a glycidylmethacrylate copolymer by reaction of PEG alkoxide with glycidyl residues [160]. This material was used as a phase transfer agent. In another emulsifier study Pitha has prepared weakened detergents for membrane studies by grafting Triton X-100 onto large hydrophilic polysaccharides such as dextran [108]. The large hydrophilic component functioned to prevent too many detergent molecules interacting with individual proteins, and also interfered with detergent entry into cells. Two routes were used for synthesis:

$$\text{RO-PEG-Cl} + \text{DEX-O}^-\text{Na}^+ \xrightarrow[60°,\ 24h]{\text{DMSO}} \text{RO-PEG-O-DEX}$$

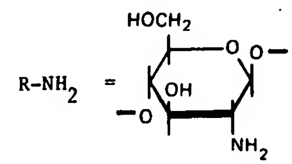
DEX = dextran





We have also attached PEG to the insoluble polysaccharide chitosan, thus making a graft copolymer soluble in many solvents [10]:

$$\text{R-NH}_2 + \text{PEG-OCH}_2\text{CHO} \xrightarrow[\text{AcOH}, H_2O, CH_3OH]{\text{NaCNBH}_3} \text{PEG-OCH}_2\text{CH}_2\text{NH-R}$$



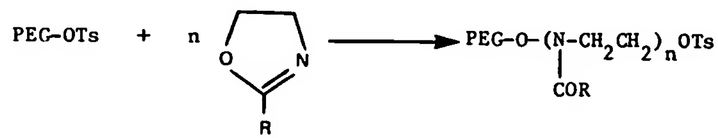
Block copolymers of PEG and two isocyanates (3,5-diisocyanato-benzylchloride or 3-nitro-3-azapentan-1,5-diisocyanate) have been prepared by Bayer for use as soluble protecting groups in peptide synthesis [40]:

$$\text{OCN—CH}_2\text{CH}_2\text{—N(NO}_2\text{)—CH}_2\text{CH}_2\text{—NCO} + \text{PEG—OH} \xrightarrow{C_6H_5CH_3}$$

$$\text{— [(—CH}_2\text{CH}_2\text{O)}_n\text{—CONH—CH}_2\text{CH}_2\text{—N(NO}_2\text{)—CH}_2\text{CH}_2\text{—NHCO}_2]_m\text{—}$$

These materials carry functional groups at defined distances all along the chain and thus make possible a high degree of substitution along the chain not possible with commercially available PEG. Bayer has also prepared similar polyurethane block copolymers for use in metal chelation and recovery [161].

Block copolymers (of ABA type) of PEG and oxazolines have been prepared by Simionescu in a process demonstrated to be a living polymerization [38]:



ACKNOWLEDGMENTS

The author gratefully acknowledges the financial support of this research by the Army Research Office and the National Aeronautics and Space Administration.

REFERENCES

[1] G. M. Powell, "Polyethylene Glycol," in *Handbook of Water-Soluble Gums and Resins*, McGraw-Hill, New York, 1980, Chap. 18.
[2] F. W. Stone and J. J. Stratta, "1,2-Epoxide Polymers," in *Encyclopedia of Polymer Science and Technology*, Vol. 6, Wiley, New York, 1967, pp. 103–145.
[3] L. E. St. Pierre, "Polymers from 1,2-Epoxides," in *Polyethers. Part I. Polyalkylene Oxides and Other Polyethers* (N. Gaylord, ed.), *High Polymers*, Vol. I, Part 1, Wiley, New York, 1963, pp. 83–291.
[4] F. E. Bailey and J. V. Koleske, *Poly(ethylene oxide)*, Academic, New York, 1976.
[5] C. P. McClelland and R. L. Bateman, *Chem. Eng. News*, *23*, 247–251 (1945).
[6] I. N. Topchieva, *Russ. Chcm. Rev.*, *49*, 494–517 (1980).
[7] H. F. Smyth, C. P. Carpenter, and C. S. Weil, *J. Am. Pharm. Assoc.*, 39, 349–354 (1950).
[8] C. G. Hunter, D. E. Stevenson, and P. L. Chambers, *Food Cosmet. Toxicol.*, 5, 195 (1967).
[9] H. F. Smyth, C. P. Carpenter, and C. S. Weil, *J. Am. Pharm. Assoc.*, *44*, 27–30 (1955).

[10] J. M Harris, M. Yalpani, J. M. Van Alstine, E. C. Struck, M. G. Case, M. S. Paley, and D. E. Brooks, *J. Polym. Sci., Polym. Chem. Ed.*, *22*, 341–352 (1984).
[11] P. Ferruti, M. C. Tanzi, L. Rusconi, and R. Cecchi, *Makromol. Chem.*, *182*, 2183–2192 (1981).
[12] G. P. Royer and G. M. Anantharmaiah, *J. Am. Chem. Soc.*, *101*, 3394–3396 (1979).
[13] A. Fradet and E. Marechal, *Polym. Bull.*, *4*, 205–210 (1981).
[14] J. M. Harris and M. G. Case, *J. Org. Chem.*, *48*, 5390–5392 (1983).
[15] K. Sukata, *Bull. Chem. Soc., Jpn.*, *56*, 280–284 (1983).
[16] G. Kopperschläger and G. Johansson, *Anal. Biochem.*, *124*, 117–124 (1982).
[17] G. Johansson, G. Kopperschläger, and P. Albertsson, *Eur. J. Biochem.*, *43*, 589–594 (1983).
[18] A. Okamoto, K. Toyoshima, and I. Mita, *Eur. Polym. J.*, *19*, 341–346 (1983).
[19] A. Abuchowski, T. van Es, N. C. Palczuk, and F. F. Davis, *J. Biol. Chem.*, *252*, 3578–3581 (1977).
[20] R. Murphy, A. C. Selden, M. Fisher, E. A. Fagan, and V. S. Chadwick, *J. Chromatogr.*, *211*, 160-165 (1981).
[21] S. Van Der Waal and L. R. Snyder, *Ibid.*, *255*, 463-474 (1983).
[22] A. M. Rothman, *Ibid.*, *253*, 283-288 (1982).
[23] C. F. Allen and L. I. Rice, *Ibid.*, *110*, 151–155 (1975).
[24] R. M. Cassidy and C. M. Niro, *Ibid.*, *126*, 787–794 (1976).
[25] W. R. Melander, A. Nahum, and C. Horvath, *Ibid.*, *185*, 129–152 (1979).
[26] T. Sivakumaran, R. T. Jenkins, W. H. C. Walker, and R. T. Goodacre, *Clin. Chem.*, *28*, 2452–2453 (1982).
[27] K. Geckeler and E. Bayer, *Polym. Bull.*, *1*, 691–695 (1979).
[28] E. Bayer, H. Zheng, and K. Geckeler, *Ibid.*, *8*, 585–592 (1982).
[29] J. M. Harris, M. G. Case, B. A. Hovanes, J. M. Van Alstine, and D. E. Brooks, *Ind. Eng. Chem., Prod. Res. Dev.*, *23*, 86–88 (1984).
[30] S. Zalipsky, C. Gilon, and A. Zilkha, *Eur. Polym. J.*, *19*, 1177–1183 (1983).
[31] H. Anzinger and M. Mutter, *Polym. Bull.*, *6*, 595–601 (1982).
[32] E. Bayer, H. Zheng, K. Albert, and K. Geckeler, *Ibid.*, *10*, 231–235 (1983).
[33] G. Ziegast and B. Pfannemuller, *Ibid.*, *4*, 467–471 (1981).
[34] M. Barelle, C. Bequin, and S. Tessier, *Org. Magn. Reson.*, *19*, 102–104 (1982).

[35] W. Schoknecht, K. Albert, G. Jung, and E. Bayer, *Liebigs Ann. Chem.*, *8*, 1514–1531 (1982).
[36] K. Bode, M. Mutter, R. P. Saltman, M. Goodman, and A. A. Ribeiro, *Biopolymers*, *22*, 163–169 (1983).
[37] D. Liebfritz, W. Mayr, R. Oekonomopulos, and G. Jung, *Tetrahedron*, *34*, 2045-2050 (1978).
[38] C. I. Simionescu and I. Rabia, *Polym. Bull.*, *10*, 311–314 (1983).
[39] K. Geckeler, *Ibid.*, *1*, 427–431 (1979).
[40] E. Bayer, I. Gatfield, H. Mutter, and M. Mutter, *Tetrahedron*, *34*, 1829–1831 (1978).
[41] *Vogel's Textbook of Practical Organic Chemistry*, 4th ed., Longmans, New York, 1978.
[42] A. F. Buckmann, M. Morr, and G. Johansson, *Makromol. Chem.*, *182*, 1379–1384 (1981).
[43] *The United States Pharmacopeia*, 15th ed., U.S. Pharmacopeia Convention, Rockville, Maryland, 1979, p. 1245.
[44] M. Mutter, *Tetrahedron Lett.*, pp. 2839–2842 (1978).
[45] L. J. Mathias, D. R. Moore, and C. B. Johnson, *J. Polym. Sci.*, *Polym. Lett.*, *21*, 711–716 (1983).
[46] D. E. Brooks and S. J. Howard, Unpublished Results.
[47] R. J. Robson and E. A. Dennis, *Acc. Chem. Res.*, *16*, 251–258 (1983).
[48] M. J. Schick (ed.), *Nonionic Surfactants*, Dekker, New York, 1967.
[49] N. Schonfeldt, *Surface Active Ethylene Oxide Adducts*, Pergamon, New York, 1969.
[50] D. Malkemus, *J. Am. Oil Chem. Soc.*, *33*, 571–574 (1956).
[51] P. N. Juri, PhD Dissertation, Texas Technological University, 1979.
[52] L. Toke, G. T. Szabo, and K. Aranyosi, *Acta Chim. Acad. Sci. Hung.*, *100*, 257–264 (1979).
[53] H. Lehmukuhl, F. Rabet, and K. Hauschild, *Synthesis*, pp. 184–186 (1977).
[54] G. Frederikson, L. Krabisch, and P. Belfrage, *J. Lipid Res.*, *23*, 1246–1248 (1982).
[55] L. Toke, G. T. Szabo, and K. Somogyi-Werner, *Acta Chim. Acad. Sci. Hung.*, *101*, 47–51 (1979).
[56] G. W. Gokel, D. M. Goli, and R. A. Schultz, *J. Org. Chem.*, *48*, 2837–2842 (1983).
[57] Y. Kimura and S. L. Regen, *J. Org. Chem.*, *48*, 195–198 (1983).
[58] J. M. Harris, N. H. Hundley, T. G. Shannon, and E. C. Struck, in *Crown Ethers and Phase Transfer Catalysis in Polymer Science* (L. Mathias and C. E. Carreher, eds.), Plenum, New York, 1984, pp. 371–384.

[59] M. A. Lukoyanova and M. M. Petukhova, *Biokhimya*, *41*, 1810–1818 (1976).
[60] P. Albertsson, *Biochemistry 12*, 2525–2530 (1973).
[61] D. J. Brunelle and D. A. Singleton, *Abstracts*, 12th Northeast Regional Meeting of ACS, Burlington, Vermont, 1982.
[62] M. D. Shalati and C. G. Overberger, *J. Polym. Sci.*, *Polym. Sci.*, *Polym. Lett. Ed.*, *20*, 473-479 (1982).
[63] F. Candau, F. Afchar-Taromi, and P. Rempp, *Polymer*, *18*, 1253–1257 (1977).
[64] B. Weiner and A. Zilkha, *J. Macromol. Sci.—Chem.*, *A11*, 1191–1200 (1977).
[65] L. J. Mathias, J. B. Canterberry, and M. South, *J. Polym. Sci.*, *Polym. Lett. Ed.*, *20*, 473–479 (1982).
[66] W. Muller, I. Hattesoho, H. Schuetz, and G. Meyer, *Nucleic Acids Res.*, *9*, 95–119 (1981).
[67] J. M. Harris, N. H. Hundley, T. G. Shannon, and E. C. Struck, *J. Org. Chem.*, *47*, 4789 (1982).
[68] M. P. Cooke, B. G. Archer, and H. Krakauer, *Biochem. Biophys. Res. Commun.*, *57*, 1032–1037 (1974).
[69] S. Akabori, M. Ohtomi, S. Masaru, and S. Ebine, *Bull. Chem. Soc. Jpn.*, *56*, 1455–1458 (1983).
[70] C. Selve, B. Castro, P. Leempoel, G. Mathis, T. Gartiser, and J. Delpuech, *Tetrahedron*, *39*, 1313–1316 (1983).
[71] E. Eriksson, P. Albertsson, and G. Johansson, *Mol. Cell. Biochem.*, *10*, 123–128 (1976).
[72] G. Johansson, *Biochim. Biophys. Acta*, *451*, 517–529 (1976).
[73] C. Axelsson and V. P. Shanbhag, *Eur. J. Biochem.*, *71*, 419–423 (1976).
[74] V. P. Shanbhag and G. Johansson, *Biochem. Biophys. Res. Commun.*, *61*, 1141–1146 (1974).
[75] G. Pinaev, A. Tartakovsky, V. P. Shanbhag, G. Johansson, and L. Backman, *Mol. Cell. Biochem.*, *48*, 65–69 (1982).
[76] R. Cecchi, L. Rusconi, M. C. Tanzi, F. Danusso, and P. Ferruti, *J. Med. Chem.*, *24*, 622–625 (1981).
[77] M. Persson, P. Stenius, L. Odberg, I. Bolmgren, H. Ljusbert-Wahren, and T. Norin, *J. Phys. Chem.*, *84*, 1557–1560 (1980).
[78] M. Mutter, H. Hagenmaier, and E. Bayer, *Angew. Chem.*, *Int. Ed.*, *10*, 811–812 (1971).
[79] V. N. R. Pillai, M. Mutter, E. Bayer, and I. Gatfield, *J. Org. Chem.*, *45*, 5364–5370 (1980).
[80] J. D. Glass, L. Silver, J. Sondheimer, C. S. Pande, and J. Coderre, *Biopolymers*, *18*, 383–392 (1979).

[81] S. Fukui, A. Tanaka, T. Iida, and E. Hasegawa, *FEBS Lett.*, *66*, 179–182 (1976).
[82] A. Okamoto, A. Hayashi, K. Uchiyama, and I. Mita, *Eur. Polym. J.*, *19*, 399–403 (1983).
[83] J. V. Karabinos and J. J. Hazdra, *J. Org. Chem.*, *27*, 4253–4255 (1962).
[84] E. Bayer and M. Mutter, *Nature*, *237*, 512–513 (1972).
[85] G. Johansson, R. Gysin, and S. D. Flanagan, *J. Biol. Chem.*, *256*, 9126–9135 (1981).
[86] A. F. Buckmann, M. Kula, R. Wichmann, and C. Wandrey, *J. Appl. Biochem.*, *3*, 301–315 (1981).
[87] P. Hubert, E. Dellacherie, J. Neel, and E. Balieu, *FEBS Lett.*, *65*, 169–174 (1976).
[88] A. Chaabouni, P. Hubert, E. Dellacherie, and J. Neel, *Makromol. Chem.*, *179*, 1135–1144 (1978).
[89] A. Chaabouni and E. Dellacherie, *J. Chromatogr.*, *171*, 135–143 (1979).
[90] S. D. Flanagan and S. H. Barondes, *J. Biol. Chem.*, *250*, 1484–1489 (1975).
[91] W. Kern, S. Iwabuchi, H. Sato, and V. Bohmer, *Makromol. Chem.*, *180*, 2539–2542 (1979).
[92] E. Ciuffarin, M. Isola, and P. Leoni, *J. Org. Chem.*, *46*, 3064–3070 (1981).
[93] T. Suzaki, Y. Murakami, and Y. Takegami, *J. Polym. Sci.*, *Polym. Lett. Ed.*, *17*, 241–244 (1979).
[94] K. Geckeler and E. Bayer, *Polym. Bull.*, *3*, 347–352 (1980).
[95] E. Boccu, R. Largajolli, and F. M. Veronese, *Z. Naturforsch.*, *38c*, 94–99 (1983).
[96] G. Johansson and A. Hartman, *Proc. Int. Solvent Extract. Conf.*, *Lyon*, pp. 927–942 (1974).
[97] F. M. Veronese, E. Boccu, O. Schiavon, G. P. Velo, A. Conforti, L. Franco, and R. Milanino, *J. Pharm. Pharmacol.*, *35*, 757–758 (1983).
[98] G. P. Royer, U.S. Patent 4,002,531 (January 11, 1977); *Chem. Abstr.*, *86*, 67539b (1977).
[99] G. Johansson, *Biochim. Biophys. Acta*, *222*, 381–389 (1970).
[100] G. Johansson, Personal Communication.
[101] F. Branstetter, H. Schott, and E. Bayer, *Tetrahedron Lett.*, pp. 2705–2708 (1974).
[102] G. Takerkart, E. Segard, and M. Monsigny, *FEBS Lett.*, *42*, 214–217 (1974).
[103] B. Weiner and A. Zilkaha, *J. Med. Chem.*, *16*, 573–574 (1973).
[104] B. Weiner, A. Zilkha, G. Porath, and Y. Grunfeld, *Eur. J. Med. Chem.—Chim. Ther.*, *11*, 525–526 (1976).

[105] B. Weiner, A. Havron, and A. Zilkha, *Isr. J. Chem.*, *12*, 863–872 (1974).
[106] B. Weiner and A Zilkha, *Eur. J. Med. Chem.—Chim. Ther.*, *10*, 79–83 (1975).
[107] R. G. Nuzzo, S. L. Haynie, M. E. Wilson, and G. Whitesides, *J. Org. Chem.*, *46*, 2861–2867 (1981).
[108] J. Pitha, K. Kociolek, and M. G. Caron, *Eur. J. Biochem.*, *94*, 11–18 (1979).
[109] E. Boccu, G. P. Velo, and F. M. Veronese, *Pharmacol. Res. Commun.*, *14*, 113–120 (1982).
[110] C. O. Beauchamp, S. L. Gonias, D. P. Manapace, and S. V. Pizzo, *Anal. Biochem.*, *131*, 25–33 (1983).
[111] A. Abuchowski, G. M. Kazo, C. R. Verhoest, Jr., T. van Es, D. Kafkewitz, A. T. Viau, and F. F. Davis, In Press.
[112] L. J. Mathias and J. B. Canterberry, *Macromolecules*, *13*, 1723–1724 (1980).
[113] W. Y. Lee and A. H. Sehon, *Immunol. Rev.*, *41*, 200–246 (1978).
[114] W. Y. Lee and A. H. Sehon, *Nature*, *267*, 618–619 (1977).
[115] T. P. King and C. Weiner, *Int. J. Peptide Protein Res.*, *16*, 147–155 (1980).
[116] K. V. Savoca, A. Abuchowski, T. van Es, F. F. Davis, and N. C. Palczuk, *Biochim. Biophys. Acta*, *578*, 47–53 (1979).
[117] K. J. Wieder, N. C. Palczuk, T. Van Es, and F. F. Davis, *J. Biol. Chem.*, *254*, 12579–12587 (1979).
[118] Y. Ashihara, T. Kono, S. Yamazaki, and Y. Inada, *Biochem. Biophys. Res. Commun.*, *83*, 385–391 (1978).
[119] A. Koide and S. Kobayashi, *Ibid.*, *111*, 659–667 (1983).
[120] Y. K. Park, A. Abuchowski, S. Davis, and F. Davis, *Anticancer Res.*, *1*, 373–376 (1981).
[121] A. Bendich, D. Kafkewitz, A. Abuchowski, and F. F. Davis, *Clin. Exp. Immunol.*, *48*, 273–278 (1982).
[122] A. Bendich, D. Kafkewitz, A. Abuchowski, and F. F. Davis, *Immunol. Commun.*, *12*, 273–284 (1983).
[123] K. Ajisake and Y. Iwashita, *Biochem. Biophys. Res. Commun.*, *7*, 1076–1081 (1980).
[124] S. D. Flanagan and D. S. Papermaster, Unpublished Results.
[125] K. A. Sharp, S. J. Howard, and D. E. Brooks, *Biochemistry*, Submitted for Publication.
[126] B. Mattiasson, T. G. I. Ling, and M. Ramstorp, *J. Immunol. Methods*, *41*, 105–114 (1981).
[127] B. Mattiasson and T. G. I. Ling, *Ibid.*, *38*, 217–223 (1980).

[128] T. G. I. Ling, M. Ramstorp, and B. Mattiasson, *Anal. Biochem.*, *122*, 26–32 (1982).
[129] J. Anzai, A. Ueno, and T. Osa, *Makromol. Chem.*, *Rapid Commun.*, *1*, 741–743 (1980).
[130] A. F. S. A. Habeeb, *Anal. Biochem.*, *14*, 328–336 (1966).
[131] P. M. Abdella, P. K. Smith, and G. P. Royer, *Biochem. Biophys. Res. Commun.*, *87*, 734–742 (1979).
[132] G. P. Royer, R. A. Liberatore, and G. M. Green, *Ibid.*, *64*, 478–484 (1975).
[133] M. Mutter and E. Bayer, *Angew. Chem.*, *Int. Ed.*, *13*, 88–89 (1974).
[134] M. Mutter, H. Mutter, and E. Bayer, *Peptides. Proceedings of the Fifth American Peptide Symposium* (M. Goodman and J. Meienhofer, eds.), Wiley, New York, 1977.
[135] C. Toniolo, G. M. Bonora, H. Anzinger, and M. Mutter, *Macromolecules*, *16*, 147–149 (1983).
[136] W. Mayer, R. Oekonomopulos, and G. Jung, *Biopolymers*, *18*, 425–450 (1979).
[137] B. Hemmasi, W. Stuber, and E. Bayer, *Hoppe-Seyler's Z. Physiol. Chem.*, *363*, 701–708 (1982).
[138] V. N. R. Pillai, *Synthesis*, pp. 1–26 (1980).
[139] V. N. R. Pillai, M. Mutter, and E. Bayer, *Tetrahedron Lett.*, pp. 3409–3412 (1979).
[140] D. Bellof and M. Mutter, *Polym. Bull.*, *11*, 49–54 (1984).
[141] F. Tjoeng, E. K. Tong, and R. S. Hodges, *J. Org. Chem.*, *43*, 4190–4194 (1978).
[142] F. Tjoeng and R. S. Hodges, *Tetrahedron Lett.*, pp. 1273–1276 (1979).
[143] M. Mutter, *Ibid.*, pp. 2843–2846 (1978).
[144] R. Colombo, *Ibid.*, pp. 4129–4132 (1981).
[145] H. Becker, H. Lucas, J. Maul, V. N. R. Pillai, H. Anzinger, and M. Mutter, *Makromol. Chem.*, *Rapid Commun.*, *3*, 217–223 (1982).
[146] A. Pollak and G. M. Whitesides, *J. Am. Chem. Soc.*, *98*, 289–291 (1976).
[147] J. M. Harris and H. Abi-Akar, Unpublished Results.
[148] B. Mattiasson, *Trends Biotechnol.*, *1*, 16–20 (1983).
[149] M. Kula, G. Johansson, and A. F. Buckmann, *Biochem. Soc. Trans.*, *7*, 1–5 (1979).
[150] M. Kula, K. H. Kroner, and H. Hustedt, *Adv. Biochem. Eng.*, *24*, 73–118 (1982).
[151] K. Geckeler and M. Mutter, *Z. Naturforsch.*, *34b*, 1024–1025 (1979).
[152] J. N. Driscoll and I. S. Krull, *Am. Lab.*, pp. 42–52 (May 1983).
[153] Y. Kimura, P. Kirszensztejn, and S. L. Regen, *J. Org. Chem.*, *48*, 385–386 (1983).

[154] S. L. Regen, *Angew. Chem.*, *Int. Ed. Engl.*, *18*, 421–429 (1979).
[155] J. G. Heffernan, W. M. MacKenzie, and D. C. Sherrington, *J. Chem. Soc.*, pp. 514–517 (1981).
[156] K. Hiratani, P. Reuter, and G. Manecke, *Isr. J. Chem.*, *18*, 208–213 (1979).
[157] H. Fujita, S. Yanagida, and M. Okahara, *Anal. Chem.*, *52*, 869–875 (1980).
[158] J. Kiji, T. Okano, H. Konishi, and Y. Nishio, *Makromol. Chem.*, *180*, 2241–2244 (1979).
[159] M. A. Twaik, M. Tahan, and A. Zilkha, *J. Polym. Sci.*, *Part A-1*, *7*, 2469–2480 (1969).
[160] J. Hradil and F. Svec, *Polym. Bull.*, *11*, 49–54 (1984).
[161] E. Bayer, P. Grathwohl, K. Geckeler, *Makromol. Chem.*, *184*, 969–976 (1983).
[162] J. R. Condor, N. A. Fruitwala, and M. K. Shingari, *J. Chromatogr.*, *269*, 171–178 (1983).
[163] S. H. Chang, K. M. Gooding, and F. E. Regnier, *Ibid.*, *120*, 321–333 (1976).

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☑ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: ______________________

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.